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DISSERTATION

Titel der Dissertation

Molecular response of the protozoan parasite *Entamoeba histolytica* to fructose as an alternative energy source and metronidazole treatment.

Verfasserin

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1. Declaration

“I declare that this doctoral thesis is my original research work and everything presented in it is a result of my own work, if not otherwise stated. Every effort was made to indicate clearly if contributions of others were involved and sources of quotations are always given.”

2. Table of contents

3. Abbreviations

µM	Micromolar
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
$CaCl_2$	Calcium chloride
CAD	Caspase-activated DNase
CPs	Cysteine proteinases
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ELISA	Enzyme linked immunosorbent assay
FEN-1	Flap endonuclease-1
Gal/GalNAc	Galactose/N-acetylgalactosamine
GDP	Guanosine diphosphate
GPI	Glucosylphosphaditylinositol
H_2O_2	Hydrogen peroxide
LINE	Long interspersed element
$MgCl_2$	Magnesium chloride
mM	Millimolar
$MnCl_2$	Manganese (II) chloride
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PFOR	Pyruvate:ferredoxin oxidoreductase
PGD2	Prostaglandin D2
Pi	Inorganic phosphate
PPGs	Proteophosphoglycans
PPi	Inorganic pyrophosphate
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROK	Receptor kinase
ROS	Reactive oxygen species
Tat	Twin-arginine translocation system
TUNEL	Terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling

4. Introduction

4.1 The parasite *Entamoeba histolytica*

The enteric protozoan parasite *Entamoeba histolytica* is the cause of amoebiasis, which typically manifests in humans as amoebic colitis or an amoebic liver abscess. The genus name "*Entamoeba*" is derived from the Greek "*ἀμοίβη* (*amoíbē*)", meaning changing shape (German: "Wechseltierchen"), whereas "*histolytica*" originates from the parasite's ability to lyse and destroy host tissues. In 1986, Walsh estimated between 36 and 50 million cases of disease and up to 110,000 deaths every year caused by this parasite. A more recent study based on data from 2010 estimated 2.24 million disability-adjusted life years (DALYs) lost annually (Hotez et al. 2014). *E. histolytica* is almost exclusively found in humans, and trophozoites cannot persist outside the human host, whereas the closely related *Acanthamoeba* spp. are free-living and do not require a host. Other important protozoan parasites found in humans include *Plasmodium* spp.*, Trypanosoma* spp.*, Leishmania* spp.*, Toxoplasma gondii, Trichomonas vaginalis and Giardia intestinalis*. *E. histolytica* has a simple lifecycle of two stages (Fig. 1). The cysts, in diameter 10-15 µm, are the infectious stage whereas the multiplying trophozoites, with a size of 10-50 µm in diameter, represent the invasive stage (Stanley 2003). Trophozoites use pseudopods for the amoeboid locomotion whereas the cysts are immobile. Fig. 2 shows trophozoites cultured in the laboratory. Mature cysts have a round shape, contain four nuclei, and are able to survive outside the human host due to their protective wall. Cysts are ingested by humans from fecally contaminated food or water and infection starts with the excystation of the cysts in the terminal ileum or the proximal colon. Eight trophozoites are released from each cyst and migrate along the colon where they can adhere to the intestinal wall and multiply by binary fission. Amoebae kill and phagocytose bacteria, human epithelial cells, immune cells and erythrocytes. Trophozoites may invade the intestinal mucosa, enter the bloodstream and reach different vital organs. Encystation occurs in the terminal colon and infectious cysts are excreted with the stool.

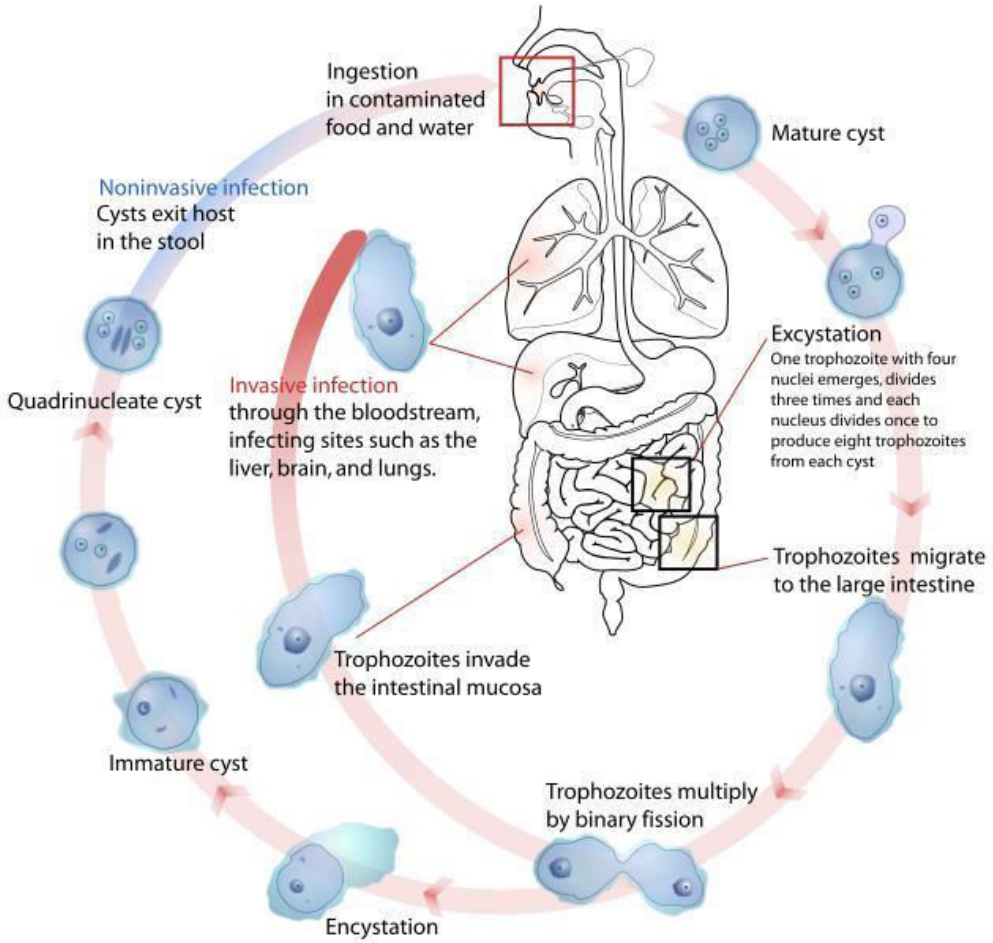

Fig. 1 *E. histolytica* life cycle
Source: Wikipedia (https://en.wikipedia. org/wiki/Entamoeba_histolytica)

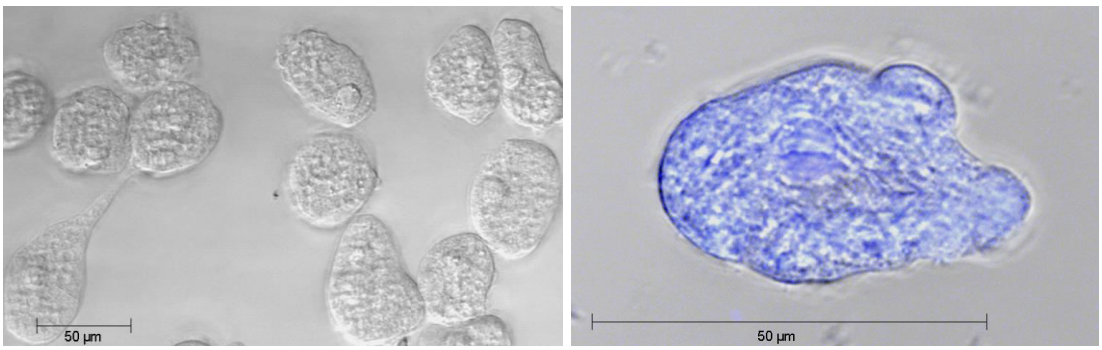

Fig. 2 *E. histolytica* trophozoites: the picture on the left shows a culture at lower magnification whereas the image on the right shows a single trophozoite at a higher resolution.

4.2 Taxonomy

Recent phylogenetic analyses of genes from various eukaryotic species revealed the Amoebozoa as a monophyletic phylum with a split into the monophyletic subphyla Lobosa and Conosa (Cavalier-Smith et al. 2015; Fig. 3). The phylum of Amoebozoa is a highly diverse major protist phylum with an estimated species number of around 2400 (Pawlowski et al. 2012). The subphylum Lobosa comprises non-flagellate amoebae whereas within Conosa, amoeboid and flagellate lineages are found. Cavalier-Smith and colleagues (2015) described with the aerobic Semiconosia and the secondarily anaerobic Archamoebae a primary dichotomy branching from Conosa. Varipodida, Dictyostelea and Entamoeba are non-flagellate Conosans which secondarily lost their cilia (Cavalier-Smith et al. 2015).

Eukaryota
- **Protozoa**
  - **Amoebozoa**
    - Lobosa
    - Conosa
      - Semiconosia
        - Mycetozoa
          - Myxomycetes
          - Dictyostelea
        - Variosea
          - Varipodida
      - Archamoebea
        - *Phreatamoeba balamuthi*
        - *Entamoeba histolytica*

Fig. 3 Taxonomic classification of *E. histolytica* (Cavalier-Smith et al. 2015).

4.3 Epidemiology

Amoebiasis is distributed all over the world but much more prevalent in tropical and subtropical areas of Africa, Asia, Central and South America. It is the third most common cause of death (after malaria and schistosomiasis) caused by a parasite (Haque 2007).

The nonpathogenic species of *Entamoeba* include *Entamoeba coli, Entamoeba hartmanni* and *Entamoeba dispar*. Whereas *E. dispar* is morphologically identical to *E. histolytica, E. coli* and *E. hartmanni* differ in their morphology. Due to the fact that *E. histolytica* can't be differentiated from the nonpathogenic *E. dispar* by microscopic examination, no differentiation between these two species was possible over many years. Consequently, the worldwide distribution of *E. histolytica* was highly overestimated in the past. Brumpt first suggested two genetically distinct species as early as 1925 but only many years later, isoenzyme analysis revealed differences in isolates from asymptomatic individuals and persons suffering from the disease (Sargeaunt and Williams 1979). From the estimated 500 million people supposed to be infected with *E. histolytica* (Walsh 1986), many people were in fact colonized by *E. dispar*.

Another important fact is that less than 10% of people infected with *E. histolytica* show an invasive disease with severe symptoms. Adults and children have equal risks of falling ill by amoebic colitis. However, significant gender differences in the demographic distribution of amoebic liver abscess are observed. This manifestation mainly affects men between the age of 18 and 50 (Stanley 2003).

Amoebiasis is still a big problem in many developing countries and parts of the world with poor hygienic conditions, but as these conditions and access to medication have improved in recent years, the disease has become much less prevalent and could be wiped out in the future.

4.4 Clinical manifestations of amoebiasis

Most common symptoms of people suffering from amoebic colitis are abdominal pain, bloody diarrhea and fatigue. Weight loss and anorexia can also occur, and in some cases, fever was reported (Adams and MacLeod 1977). The most frequent extraintestinal manifestation of an *E. histolytica* infection is

a liver abscess. In patients with amoebic liver abscess, often no trophozoites or cysts can be found in stool, and bowel symptoms are rarely present. Possible symptoms include fever, hepatic tenderness, right upper quadrant pain and cough as well as weight loss in chronic cases (Adams and MacLeod 1977). Seven to 20% of people who developed a liver abscess suffered from pleuropulmonary amoebiasis thereafter. A rare outcome is an amoebic brain abscess (encephalitis), which is, again, mainly observed in patients suffering from amoebic liver abscess.

4.5 Diagnosis

The classical method to diagnose amoebic colitis is the microscopic examination of the stool samples for *E. histolytica* trophozoites or cysts. However, the sensitivity of this method is not very high, and several stool samples need to be taken. In addition, it is not possible to distinguish morphologically between *E. histolytica* and the nonpathogenic *E. dispar*. Therefore, alternative methods for the diagnosis of an *E. histolytica* infection were established. The oldest method, once the gold standard, was to culture the amoebae and to perform isoenzyme electrophoresis (Sargeaunt et al. 1978). This laborious method was replaced by ELISA tests which can identify antigens in stool and also differentiate between *E. histolytica* and *E. dispar* (Pillai et al. 1999; Haque et al. 2000). The current diagnostic tool to determine an *E. histolytica* infection is to use genetic differences (Clark and Diamond 1993; Mirelman et al. 1997) and to perform real-time PCR assays (Blessmann et al. 2002). For the diagnosis of an amoebic liver abscess, a computed tomography scan or ultrasonography is the method of choice. In addition, amoebic serology should be performed (Stanley 2003). As described above, stool tests play a smaller role.

4.6 Treatment

Little more than half a century ago, amoebiasis used to be a fatal disease, but after the introduction of effective medical treatment, mortality rates dropped significantly (Stanley 2003).

For the treatment of amoebiasis, the nitroimidazole drug metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) has been used for almost 50 years (Powell et al. 1966). As no relevant resistance against metronidazole has

been reported until now, it is still used as the gold standard drug for the treatment of an amoebic liver abscess. In the case of amoebic colitis, a luminal agent (paromomycin, iodoquinol, or diloxandide furoate) is used in addition to metronidazole to get rid of amoebic intestinal colonization (Pehrson and Bengtsson 1984). Asymptomatic patients should also take such a luminal agent, as these persons are in danger of developing an invasive disease and also represent a risk for public health.

4.7 Pathophysiology – the amoebic attack against human cells

Amoebic colitis causes tissue damage and inflammation in the colon. The amoebic adherence to the target cells is mediated through the galactose/N-acetylgalactosamine (Gal/GalNAc) specific lectin (Petri and Mann 1993; Petri et al. 2002). *E. histolytica* trophozoites do not attach to mammalian cells without surface Gal or GalNAc residues, and they do not kill such cells (Stanley 2003).

Up to this date, research is ongoing on how the host cells are killed by the amoebae. A major role is played by the so-called “amoebapores”, small amphipathic polypeptides forming pores in lipid bilayers (Leippe 1997). These pores allow the free flow of ions through the plasma membrane leading to a breakdown of the membrane potential.

Huston and colleagues (2003) described an apoptotic host cell-killing followed by phagocytosis of these cells by the amoebae. Activated caspase 3 was found in almost all intact Jurkat cells ingested by *E. histolytica*. In a mouse model, it was found that apoptosis plays an important role for the formation of an amoebic liver abscess, shown via the inhibition of caspases (Yan and Stanley 2001). In an earlier study, necrotic cell death was reported in HL-60 and Jurkat leukemia cells (Berninghausen and Leippe 1997). In this study, typical signs of apoptotic cell death, such as blebbing, shrinkage of the cell, or apoptotic DNA degradation were absent.

As described above, previous studies suggested that human cells are killed first and ingested afterwards (Huston et al. 2003; Ralston and Petri 2011), however, in a recent study, it was shown that only fragments of living cells were engulfed by the parasite, which finally led to the death of the cells (Ralston et al. 2014). This process was termed trogocytosis.

Moreover, *E. histolytica* cysteine proteinases (CPs) play an important role in invasion, inflammation and tissue damage of the human intestine. These proteinases digest extracellular matrix proteins (Scholze and Werries 1986; Keene et al. 1986), so amoebae can invade. Kelsall and Ravdin (1993) reported a complete degradation of human IgA molecules by *E. histolytica* and suggested CPs as being responsible for this degradation. *E. histolytica* has more active CP genes than *E. dispar* (Bruchhaus et al. 1996), and CP5 is specifically present on the surface of *E. histolytica* (Jacobs et al. 1998). Overexpression of some CPs in a nonpathogenic *E. histolytica* clone restored its pathogenicity (Matthiesen et al. 2013). In addition to its own proteinases, *E. histolytica* is able to induce matrix metalloproteinases in the human host, which increases tissue destruction (Thibeaux et al. 2014).

Besides causing amoebic colitis, the parasite can invade the liver, lyse hepatocytes and cause massive cell death, with the result of an amoebic liver abscess. Hepatocytes are a good iron source and as iron is an important metal cofactor for amoebae (Espinosa et al. 2009), human cells with iron containing proteins are essential for the parasite (López-Soto et al. 2009).

4.8 Immunology – the host response to the invading amoebae

Lysis of human epithelial cells mediates the release of interleukin-1α and interleukin-1β precursor. Moreover, interleukin-8 and cyclooxygenese-2 are produced. The release of cytokines and chemokines attracts neutrophils and macrophages to the site of invasion. However, the neutrophils are not able to eliminate the invading trophozoites, and are killed themselves. As a consequence, more mediators might be released, which cause more damage to the intestinal epithelium and increased inflammation (Stanley 2003).

Macrophages, especially when activated, are able to kill amoebic trophozoites. A recent study showed that direct contact of amoebae via the Gal/GalNAc specific lectin and with the involvement of CP5 led to the triggering of the macrophage inflammasome resulting in a strong inflammatory response (Mortimer et al. 2015). Macrophages can also present antigenic peptides from killed amoebae to lymphocytes. As a result, antibodies against the parasite are produced. Human IgA is secreted at mucosal sites and as the

major systemic antibody, IgG is produced. As an evasion mechanism, the amoebae are able to cap antibodies bound to their surface and to shed them from their posterior end, the uroid (Calderon et al. 1980).

The human complement system represents another humoral defense mechanism against the invasion of *E. histolytica* trophozoites. However, amoebae show resistance to human complement components, such as the C5b-9 membrane attack complexes. The CD59 antigen on human blood cells is a membrane inhibitor of the C5b-9 complex. Braga and colleagues (1992) showed that the *E. histolytica* Gal/GalNAc lectin contains a region with similarity to the complement-inhibitory CD59.

Glycosylphosphaditylinositol (GPI)-anchored proteophosphoglycans (PPGs) represent major surface antigens (Moody-Haupt et al. 2000). On the one hand they may protect the surface of the amoebae, on the other hand they activate toll-like receptors 4 and 2 stimulating the innate immune response (Maldonado-Bernal et al. 2005). Moreover, a monoclonal antibody against PPGs protected SCID mice from amoebic liver abscess (Marinets et al. 1997).

Taken together, there is a balance between the strategies of the human host and the amoebae. Although generally a protective response develops after an amoebic disease, a later episode of the disease cannot be fully prevented.

4.9 Metabolism

4.9.1 Overview

Many metabolic pathways in *E. histolytica* have been investigated on the biochemical level, revealing for example that amoebae lack a functional Krebs cycle and oxidative phosphorylation (Reeves 1984).

E. histolytica lives in an environment with low oxygen concentrations, and therefore has to gain energy from anaerobic conversion of glucose via pyruvate to ethanol (Reeves 1984), or from degradation of amino acids (Clark et al. 2007). The organism lacks mitochondria, most likely due to secondary loss (Clark and Roger 1995). Instead, an organelle named mitosome is found in *E. histolytica,* which is almost certainly derived from mitochondria. Mitosomes were characterized as the most reduced mitochondrion-related organelles that do not generate energy (Makiuchi and Nozaki 2014). So far, mitosomes were

only found in anaerobic and microaerophilic organisms that do not possess mitochondria.

The *E. histolytica* genome sequencing project revealed some new insights into metabolic processes in this parasite. The international project was performed by Brendan Loftus and colleagues at the Institute for Genomic Research (TIGR), Neil Hall and colleagues at the Sanger Institute, and different other working groups from all over the world (Loftus et al. 2005). The haploid *E. histolytica* genome was described to consist of around 24 mega basepairs, which is quite small compared for example to the genome of trichomonads. In the initial annotation, 9938 genes were predicted. A later reannotation of the genome (Lorenzi et al. 2010) reduced the genome size to 20 mega basepairs and the number of genes to 8201.

In entamoebae, many amino acid biosynthetic pathways are missing, purine and pyrimidine synthesis are not found, and nucleotide reductase is also missing (Anderson and Loftus 2005; Loftus et al. 2005).

4.9.2 The glycolysis (Embden-Meyerhof-Parnas) pathway

In the absence of oxidative phosphorylation in mitochondria, much less ATP can be generated from glucose. *E. histolytica* possesses a classical glycolysis (Embden-Meyerhof-Parnas) pathway from glucose to pyruvate (Fig. 4) with some special features: phosphofructokinase uses PPi rather than ATP, saving the investment of one ATP molecule, and phosphoglycerate kinase uses GDP instead of ADP. In addition, an alternative to the pyruvate kinase is found in *E. histolytica*, pyruvate orthophosphate dikinase. Phosphoenolpyruvate plus AMP and PPi is converted to pyruvate plus ATP and Pi.

In the final stage of glucose utilization, pyruvate is converted to acetyl-CoA and carbon dioxide by pyruvate:ferredoxin oxidoreductase (PFOR) (Reeves 1984). Under strictly anaerobic conditions, Acetyl-CoA is then reduced to ethanol by a bifunctional alcohol/aldehyde dehydrogenase with the regeneration of NAD^+. In the presence of small amounts of oxygen, the amoebae have alternatives to produce NAD^+ (Weinbach and Diamond 1974). In the so-called aerobic metabolism, an additional molecule of ATP is generated via the conversion of acetyl-CoA to acetate, which is then secreted.

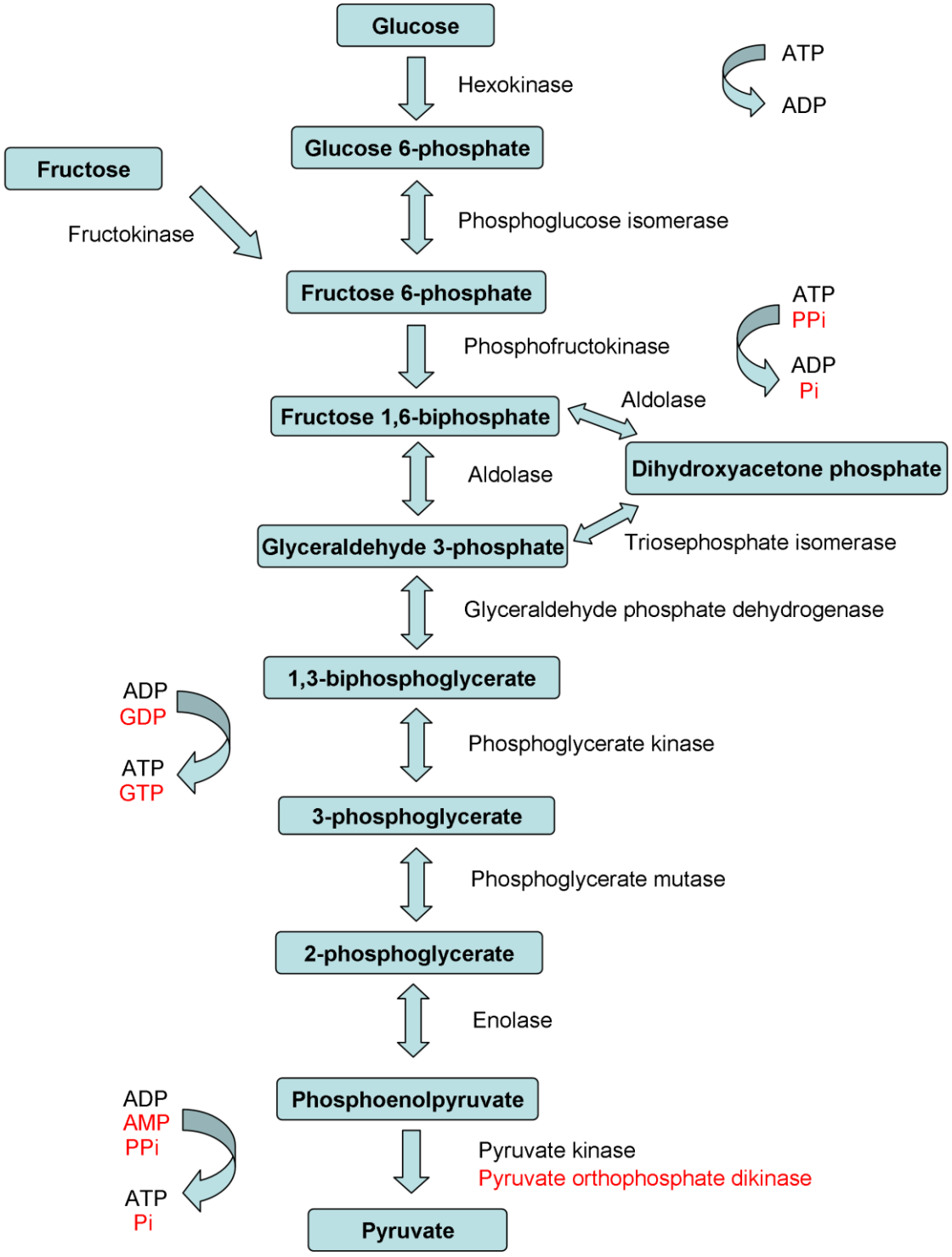

Fig. 4 The glycolytic pathway in *E. histolytica*. Some important differences compared to other organisms are highlighted in red.

4.9.3 Metabolic stress – deprivation of nutrients

With a parasitic lifestyle, *E. histolytica* depends on a supply of nutrients from the host, so nutrient deprivation could play an important role. Only scarce information about the effects of metabolic stress in amoebae exists. The effect of glucose starvation on the virulence of *E. histolytica* was investigated *in vitro* by Tovy and colleagues (2011), who reported that the parasite's virulence is boosted by glucose starvation. However, downregulation of the virulence factors amoebapore A and cysteine proteinase A5 suggested that these two factors are not essential for the virulence of glucose-starved trophozoites. Further, resistance to heat shock or oxidative stress was similar in control amoebae and under starvation (Tovy et al. 2011). Moreover, increased virulence and alteration of transcription of several genes in *E. histolytica* grown in medium without glucose was reported by Baumel-Alterzon and colleagues (2013). In their study, they described the special role of the dihydropyrimidine dehydrogenase gene, which is involved in pyrimidine metabolism, and is essential for the adaptation of amoebae growing under glucose starvation.

4.9.4 Metabolic stress – fructose as an alternative energy source

The question addressed in the first part of this work was how the amoebae could respond to an abrupt change in the main nutrient. Media used for the axenic cultivation of *E. histolytica* trophozoites contain glucose (Diamond et al. 1978, 1995), which is readily taken up and metabolized by the amoebae (Serrano and Reeves 1974). In the first step of glycolysis, glucose is phosphorylated by one of the two hexokinases present in this parasite (Ortner et al. 1995). However, in the human host, glucose will never be found in concentrations as high as in culture media (10 g/l). In the colon, amoebae encounter only low levels of glucose (~ 0.2 g/kg tissue; Baumel-Alterzon et al. 2013). In contrast, fructose may be found in higher concentrations, especially in case of a fructose malabsorption trait, which is commonly found in humans (Latulippe and Skoog 2011). Therefore, in case of a lack of glucose, fructose could represent an alternative nutrient to gain ATP. The two *E. histolytica* hexokinase isoenzymes phosphorylate glucose well but they are not able to phosphorylate fructose (Kroschewski et al. 2000). This stands in contrast to human hexokinases, which are able to phosphorylate fructose (Middleton 1990).

In glycolysis (Fig. 4), intermediates provide entry points for many monosaccharides, and fructose could be converted into one of these intermediates, fructose 6-phosphate, through the enzyme fructokinase.

In the *E. histolytica* genome (Loftus et al. 2005*)*, a fructokinase gene with similarity to bacterial fructokinases can be identified. The enzyme belongs to the ribokinase family, which is characterized as being involved in carbohydrate transport and metabolism. Generally, kinases can be grouped into 4 non-homologous families: galactokinases, hexokinases, ribokinases (Bork et al. 1993), and a novel family of receptor kinases (ROK). Based on amino acid sequence similarities, proteins with bacterial repressors, uncharacterized open reading frames and sugar kinases were grouped together to this family (Titgemeyer et al. 1994).

A BlastP search in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed closest relation of the *E. histolytica* fructokinase to *Prevotella maculosa*, after the genus *Entamoeba*. Therefore, this gene is a good candidate where lateral gene transfer from bacteria might have occurred (Loftus et al. 2005). This event may have been an early one, as all *Entamoeba* spp. sequenced up to this date possess homologs of this bacterial-type fructokinase gene. Especially some genes coding for metabolic enzymes could have been gained early by lateral gene transfer from bacteria (Loftus et al. 2005; Clark et al. 2007).

4.9.5 Strong metabolic stress – redox stress through oxygen, reactive oxygen and nitrogen intermediates

Besides nutrient stress, the activity of the human immune system can generate strong metabolic stress in *E. histolytica*. Upon host infection, *E. histolytica* is exposed to reactive oxygen and nitrogen species (ROS and RNS), which requires active responses in this microaerophilic parasite. Amoebae lack most of the usual anti-oxidative defense mechanisms found in many eukaryotic species (Mehlotra 1996; Loftus et al. 2005), therefore, alternative strategies must be present in this parasite. As a response to oxidative or nitrosative stress, several genes were found to be differentially expressed, surprisingly, the transcription of genes encoding enzymes that neutralize ROS and RNS was generally not increased upon oxidative stress (Vicente et al. 2009). Some

enzymes involved in energy metabolism are inactivated due to oxidative stress (Pineda et al. 2010). Generally, the genome of *E. histolytica* encodes a number of proteins responsible for the detoxification of ROS and RNS (Loftus et al. 2005). Pearson and colleagues (2013) found 57 genes with increased expression levels in response to H_2O_2 exposure. Thereafter, they characterized a novel transcription factor (accession number EHI_108720) which mediates up-regulation of gene expression upon oxidative stress. The parasite's virulence was increased after overexpression of this transcription factor. Another recent study on oxidative stress in *E. histolytica* trophozoites showed glycolytic inhibition and the redirection of metabolic flux towards glycerol production, chitin biosynthesis and the non-oxidative branch of the pentose phosphate pathway (Husain et al. 2012). Therefore, the glycerol biosynthetic pathway was proposed to play an important role in the anti-oxidative defense system in *E. histolytica*. Gosh and colleagues (2010) reported that 42% of *E. histolytica* trophozoites showed DNA fragmentation when oxidative stress was induced.

Nitric oxide is supposed to be the major cytotoxin responsible for the killing of amoebae; released by phagocytic cells, activated macrophages and natural killer cells (Lin and Chadee 1992; Hertz et al. 2014). Many key enzymes can be inhibited via its reactivity as an S-nitrosylating agent. Gene expression analysis showed that stress responses were triggered by nitric oxide. This potent cytotoxin directly inhibits glycolysis and raises cysteine synthase activity in *E. histolytica* (Santi-Rocca et al. 2012). In nitric oxide exposed trophozoites, 142 S-nitrosylated proteins were detected which are involved in glycolysis, gluconeogenesis, protein transport, translation and adherence to target cells (Hertz et al. 2014).

Iron chelation is another factor which can affect the survival of amoebae. Alcohol dehydrogenase represents an important enzyme for the glycolytic pathway of *E. histolytica* and iron is an essential cofactor. Iron starvation caused an interruption in the glycolytic pathway as the enzymatic activities of alcohol dehydrogenase and aldehyde dehydrogenase were directly inhibited by iron chelation (Espinosa et al. 2009).

4.9.6 Severe metabolic stress – metronidazole action

Metronidazole is used for the treatment of infections caused by microaerophilic protozoan parasites such as *E. histolytica*, *Giardia intestinalis* or *Trichomonas vaginalis*, and bacteria such as *Bacteroides* spp., *Helicobacter pylori* or *Clostridium difficile*. Up to date, there are no reports of clinically relevant resistances of *E. histolytica* against this drug.

Although metronidazole has been used successfully for almost half a century, it is not quite clear how this drug kills the amoebae. It is undisputed that metronidazole is a prodrug which must be reduced at its nitro group to become active. Preferentially, this reduction occurs in anaerobic and microaerophilic organisms, rather than in the aerobic host. The 5-nitro group of metronidazole is first reduced to the nitroradical anion (Müller 1983), which is further reduced to the intermediates carrying nitroso- and hydroxylamine groups, and finally to the amino-derivative, which is less toxic. In the presence of oxygen, as it is the case in host cells, the nitroradical anion can be reoxidized to the nontoxic parent compound. This process, called “futile cycling” (Mason and Holtzman 1975), generates superoxide anions, causing oxidative stress for the microaerophilic parasite, but much less for the human host cells.

There are several theories about how metronidazole is activated. The classical theory suggests that the PFOR reaction generates reduced ferredoxin, which activates metronidazole. In an alternative way, thioredoxin reductase was found to catalyze the NADPH-dependent reduction and activation of metronidazole in *E. histolytica* (Leitsch et al. 2007), as well as in *T. vaginalis* (Leitsch et al. 2009). Finally, Jeelani and colleagues (2010) identified two L-cysteine-regulated NADPH-dependent oxidoreductases with possible metronidazole-reducing activity.

Thioredoxin reductase does not only activate metronidazole, it is also a target of this nitroimidazole drug. Some *E. histolytica* proteins form covalent adducts with activated metronidazole and other nitroimidazole drugs. The identified modified proteins were thioredoxin reductase itself, thioredoxin, superoxide dismutase, purine nucleoside phosphorylase and the novel protein metronidazole target protein-1 (Leitsch et al. 2007). For the repair of oxidatively damaged proteins, the thioredoxin reductase/thioredoxin system is very important, and the covalent modification by metronidazole inhibits this activity (Leitsch et

al. 2007). In *E. histolytica*, more than 200 proteins were found to be able to interact with thioredoxin (Schlosser et al. 2013). Although the important thioredoxin system is the target of metronidazole, this cannot fully explain its mode of action, as the drug is also active in the absence of oxidative stress.

DNA damage was one central observation in metronidazole-treated protists. The classical theory maintained that the nitroradical anion, the initial product of metronidazole reduction, damages proteins and DNA (Müller 1983; Edwards 1977, 1993). The destruction of DNA was seen as a major part of metronidazole activity. However, LaRusso and colleagues (1977) observed no DNA damage from reduced metronidazole *in vitro*. The hypothesis of DNA degradation by the nitroradical anion is also challenged by the low reactivity of this radical, which has raised doubts about its ability to break DNA strands (Wardman 1985). These doubts were strengthened by the fact that DNA degradation is also observed when amoebae are treated with non-radical forming agents, such as G418 (Villalba et al. 2007). This led to the alternative hypothesis that the DNA is not directly damaged by the activated drug, but through the induction of cellular DNase activity. A significant part of this thesis was devoted to the search for such an activity in *E. histolytica*.

4.10 Programmed cell death (PCD)

4.10.1 PCD in multicellular organisms

In multicellular eukaryotic organisms, some cells have to be sacrificed for the maintenance of tissue homeostasis and when cells are damaged or infected. This does not happen in a random way, it is an active and energy dependent genetically determined process. This process follows various intracellular and extracellular stimuli and was termed programmed cell death (PCD). A formal classification differentiates between two types of PCD: type I PCD (apoptosis) and type II PCD (autophagic cell death) (Schweichel and Merker 1973; Clarke 1990; Bruchhaus et al. 2007). Many recent studies revealed a highly complex picture of regulated cell death (reviewed in Galluzzi et al. 2015).

The apoptotic process is a mechanism resulting in cellular and biochemical changes. Many characteristic morphological alterations are part of this process, such as cytoplasmic contraction, cell shrinkage, chromatin condensation, and plasma membrane blebbing. On the molecular level, changes include increased intracellular calcium levels, low ATP levels and externalization of phosphatidylserine. DNA fragmentation, discussed in detail below, is a major and characteristic feature of apoptosis. The main apoptotic signaling occurs through the activation of a series of caspases (aspartate-specific cysteine proteases; Cohen 1997). Even though apoptosis is an evolutionary conserved mechanism, many inter- and intra-species specific variations can be observed (Duque-Parra 2005).

Autophagy is a process that is induced by cells when they undergo phases of starvation or oxidative stress. Cells recycle cell material and digest parts of their own cytosolic material, including organelles. This mechanism provides energy and cells might even be able to avoid cell death (Levine and Yuan 2005).

Necrosis is, in contrary to PCD, no active process; it is not genetically determined and does not require energy. Cell death can be caused by inflammation, cancer or infection. Typical characteristics of necrotic cell death include a gain in cell volume, swelling of organelles and plasma membrane permeabilization.

4.10.2 PCD in unicellular organisms

In the past, apoptosis was thought to be present in multicellular organisms only, but during the last two decades, more and more reports demonstrated this phenomenon in unicellular eukaryotes as well. Although there is no obvious advantage of PCD for unicellular organisms, one theory suggests that it could be related to altruistic behavior, which benefits the entire population (Wanderley et al. 2005).

Several studies described various features of PCD in protozoan parasites. So far, PCD was analyzed in *Trypanosoma* spp.*, Leishmania* spp.*, Plasmodium* spp.*, G. intestinalis, Blastocystis hominis, T. vaginalis,* and *E. histolytica* (reviewed in Bruchhaus et al. 2007 and Jiménez-Ruiz et al. 2010). Programmed cell death in *E. histolytica* has been described with different inducers of cell death: nitric oxide species (Ramos et al. 2007), hydrogen peroxide (Ghosh et al. 2010, Nandi et al. 2010), and the aminoglycoside G418 (Villalba et al. 2007). There are only preliminary PCD studies with metronidazole as cell death inducer in *E. histolytica* (Seifert, unpublished data). However, apoptotic-like death of protozoan parasites upon exposure to metronidazole has been reported in *B. hominis (*Nasirudeen et al. 2004) and *G. intestinalis* (Ghosh et al. 2009; Bagchi et al. 2012).

Apoptotic characteristics, such as cytoplasmic vacuolisation, membrane blebbing and nuclear chromatin condensation, were first observed in *Trypanosoma cruzi* (Ameisen et al. 1995) and *Trypanosoma brucei* (Welburn et al. 1996). Externalisation of phosphatidylserine has been reported in a number of species (Jiménez-Ruiz et al. 2010), including *E. histolytica* (Ghosh et al. 2010; Seifert, unpublished data). These morphological signs were always accompanied by degradation of DNA, usually measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays.

Whereas main features of damage are similar in PCD of multi- and unicellular organisms, there are large differences concerning the regulation. Activation of caspases is most important for the regulation of apoptotic processes in metazoans. Protease activities and caspase-like activities have been reported in protozoans, however, no real caspase has been identified so far, and caspase inhibitors were not able to prevent apoptosis in protozoans (Jiménez-Ruiz et al. 2010). Paracaspases and metacaspases represent two related protein

families, metacaspases were found in the protozoan parasites *Plasmodium, Trichomonas, Trypanosoma, Leishmania* and *Acanthamoeba*, but not in *Giardia* and *Entamoeba.*

4.10.3 DNA degradation during programmed cell death

As indicated above, one of the hallmarks of apoptosis, and probably the most frequent marker, is DNA fragmentation. Generally, high molecular weight DNA cleavage is observed at the beginning of apoptotic processes, whereas low molecular weight DNA cleavage occurs later, resulting in the so-called DNA ladder, which is typically observed in apoptotic cells (Samejima and Earnshaw 2005). During apoptosis, caspase-activated DNase (CAD) is an important nuclease responsible for chromatin condensation and DNA fragmentation (Widlak and Garrard 2005). CAD is evolutionary conserved from flies to mammals (Table 1); however, lower eukaryotes lack this major apoptotic nuclease (Samejima and Earnshaw 2005), as they lack caspases as well.

Another evolutionary conserved nuclease is endonuclease G. This major apoptotic nuclease is localized in mitochondria and can induce DNA cleavage in cells lacking a CAD system. Moreover, activation of caspases is not necessary for the translocation of endonuclease G from mitochondria to the nucleus when apoptosis is induced (Li et al. 2001). Nucleases similar to endonuclease G were recently described in *Leishmania* (Bosedasgupta et al. 2008; Rico et al. 2009) and in *T. brucei* (Gannavaram et al. 2008) (Table 1), but no endonuclease G is found in *E. histolytica*, not surprisingly, as *E. histolytica* lacks mitochondria.

The ubiquitous FEN-1 (flap endonuclease-1) is a further major endonuclease which can play a role during apoptosis. Normally, FEN-1 cuts flaps (short single-stranded DNA overhangs) during DNA replication and repair (Tsutakawa et al. 2014). In *E. histolytica,* whole genome analysis has revealed a homolog of FEN-1 (Table 1).

TatD was first discovered in *Escherichia coli* as an open reading frame in the *tat* (twin arginine transport) operon (Sargent et al. 1998), and in 2000, Wexler and colleagues described a DNase activity of the bacterial TatD protein. This nuclease represents a protein conserved across kingdoms and is characterized in several species as an apoptotic or apoptotic-like nuclease. TatD homo-

logs involved in DNA degradation were identified in *E. coli* (Wexler et al. 2000), *Caenorhabditis elegans* (Parrish and Xue 2003), *Saccharyomyces cerevisiae* (Qiu et al. 2005), *Leishmania donovani* (BoseDasgupta et al. 2008) and *T. brucei* (Gannavaram and Debrabant 2012). Accordingly, a homolog of the bacterial TatD nuclease was identified in *E. histolytica* (EHI_119490, XP_651470). As TatD was the only DNase in this organism expected to cut intact DNA, it was conceivable that it could play a role in the metronidazole-induced DNA degradation, and therefore *E. histolytica* TatD was investigated in detail.

Table 1 Presence (+) and absence (-) of various DNase candidates for the involvement in apoptosis from prototype organisms.

Apoptotic DNases	**Human**	***C. elegans***	***S. cerevisiae***	***Drosophila***	***T. brucei***	***Leishmania***	***Giardia***	***E. histolytica***
DFF40/CAD	+	-	-	+	-	-	-	-
DNase II	+	+	+	+	-	-	-	-
Endonuclease G	+	+	+	+	+	+	-	-
FEN I	+	+	+	+	+	+	+	+
TatD	+	+	+	+	+	+	+	+

5. Aims of the thesis

The overall aim of the present thesis was to examine the response of the parasite *Entamoeba histolytica* to chemical stress. The work addressed two main examples:

First of all, a closer look was taken at the consequences of nutrient deprivation in *E. histolytica*. Addressing this problem, fructose was considered as an alternative energy source for amoebae in the human colon. Thus, major focus was directed towards the putative *E. histolytica* fructokinase, as the functional enzyme is essential for the introduction of fructose into glycolysis, allowing the generation of ATP from fructose fermentation. For this part of the study, the aim was to examine the growth of amoebae in a medium containing fructose instead of glucose, to clone the *E. histolytica* fructokinase, to express it in *E. coli,* and to analyze the fructokinase mRNA expression of cells grown with fructose medium. In addition, a biochemical characterization of the enzyme was planned as well as its localization in the amoebae by confocal immunofluorescence microscopy.

In metronidazole-treated *E. histolytica*, DNA damage can be observed *in vitro*, but it is not quite clear if this DNA fragmentation is the cause or the consequence of the breakdown of cellular processes. As described earlier, the classical theory suggests that DNA damage in the parasite is caused by the nitro-radical anion. Our hypothesis suggests that DNA degradation is caused by nucleases from the parasite, induced in response to the metronidazole treatment. However, so far, no DNase has been described in *E. histolytica* and no genes coding for classical DNases I or II, or apoptotic nucleases such as CAD (caspase-activated DNase) or endonuclease G were found in the database. Therefore, another aim of this thesis was the identification and characterization of an *E. histolytica* DNase which could be responsible for DNA destruction after metronidazole treatment.

TatD, a protein originally described in *E. coli*, was the only DNase with a possible role in apoptotic DNA degradation present in *E. histolytica*. Thus, this protein was of special interest for us. It was planned to clone and express *E. histolytica* TatD in *E. coli*, to investigate its activity and cellular localization as

well as the expression of the mRNA encoding TatD in amoebae treated with metronidazole. In addition, it was planned to examine DNA fragmentation in amoebae in general as well as the mRNA expression of other putative nuclease genes not linked to apoptosis-like cell death in response to metronidazole treatment.

6. Overview of peer reviewed publications/manuscripts

Molecular and biochemical characterization of *Entamoeba histolytica* fructokinase (Chapter 7)

Author names:

Julia Matt, Michael Duchêne

Reference:

Parasitology Research (2015) 114: 1939-1947.

doi: 10.1007/s00436-015-4383-5

Author contributions:

The experiments were designed by JM and MD. JM performed the analyses, and JM and MD wrote the manuscript.

***Entamoeba histolytica*: Molecular Characterisation of a DNase Homologous to Bacterial TatD (Chapter 8)**

Author names:

Julia Matt, Sarah Schlosser, Nancy Guillén, Michael Duchêne

Reference:

This manuscript was submitted to PLOS Neglected Tropical Diseases on 2014 Aug 7

Author contributions:

The experiments were designed by JM, SS, NG and MD. JM performed the analyses, and JM and MD wrote the manuscript.

7. Molecular and biochemical characterization of *Entamoeba histolytica* fructokinase

Parasitol Res (2015) 114:1939–1947
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ORIGINAL PAPER

Molecular and biochemical characterization of *Entamoeba histolytica* fructokinase

Julia Matt · Michael Duchêne

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Abstract *Entamoeba histolytica* is the causative agent of amoebic dysentery and liver abscess. The medium for its axenic culture contains glucose as energy source, and we addressed the question whether *E. histolytica* can also use fructose instead. As the amoebic hexokinases do not phosphorylate fructose, a separate fructokinase is essential. The genome project revealed a single candidate gene encoding an *E. histolytica* homolog of bacterial fructokinases. This gene was cloned, and the recombinant enzyme had a magnesium-dependent fructose 6-kinase activity (EC 2.7.1.4) with a K_m for fructose of 0.156 mM and a V_{max} of 131 U/mg protein. Recombinant fructokinase also showed a much weaker mannokinase activity, but no activity with glucose or galactose. The amoebae could be switched from glucose to fructose medium without any detectable consequence on doubling time. Fructokinase messenger RNA (mRNA) was modestly but significantly upregulated in amoebae switched to fructose medium as well as in fructose-adapted *E. histolytica*.

Keyword *Entamoeba histolytica* · Glucose · Fructose · Fructokinase · Hexokinase

Introduction

The protozoan parasite *Entamoeba histolytica* is the cause of amoebic dysentery and liver abscess. In an older study (Walsh 1986), between 36 and 50 million cases of disease and up to 110,000 deaths per year were estimated, whereas in a recent assessment of the situation in 2010, the disease burden of amoebiasis was estimated at 2.24 million disability-adjusted life years (DALYs) lost annually (Hotez et al. 2014).

The intestinal parasite exists in a microaerophilic environment and lacks a functional Krebs cycle, mitochondria and oxidative phosphorylation, so glycolysis is the major source of energy (Reeves 1984). Accordingly, the two most commonly used media for the axenic culture of *E. histolytica*, TYI-S-33 (Diamond et al. 1978) and YI-S (Diamond et al. 1995) both contain glucose as the major energy source. Glucose is readily taken up (Serrano and Reeves 1974) and phosphorylated by one of the two hexokinases (Ortner et al. 1995) as the first step of glycolysis. These two steps together with glycogen breakdown were found to have the largest influence on the glycolytic flux (Pineda et al. 2014).

In the human host, under normal conditions, almost 100 % of the glucose is absorbed before it reaches the colon and the amoebae never encounter the glucose concentration provided in the culture media. In contrast, fructose may be found at varying but sometimes significant concentrations, at least in case of fructose malabsorption, which is a common trait (Latulippe and Skoog 2011).

Although *E. histolytica* can tolerate only modest oxygen concentrations, the organism is able to consume oxygen and its uptake is strongly stimulated by glucose (Weinbach and Diamond 1974). The glycolytic pathway from glucose to acetyl-CoA generates NADH. To regenerate NAD^+, NADH can be used to reduce acetyl-CoA to ethanol, or NADH can be transformed to NADPH which can reduce oxygen eventually to H_2O. Thus, acetyl-CoA can be spared for the generation of an extra molecule of ATP, and this type of aerobic metabolism provides a small benefit for the amoebae.

In *E. histolytica*, fructose stimulated the aerobic metabolism at only 30 % of the level of glucose (Weinbach and

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Diamond 1974); moreover, the two hexokinase isoenzymes of *E. histolytica* were unable to phosphorylate fructose (Kroschewski et al. 2000), unlike the hexokinases of the human host (Middleton 1990). On the other hand, the *E. histolytica* genome (Loftus et al. 2005) contains a gene coding for a sugar kinase with similarity to bacterial fructokinases. It was hypothesized that this gene was acquired from bacteria by lateral gene transfer (Loftus et al. 2005).

In general, sugar kinases can be grouped into non-homologous families: Two large families of hexokinases and ribokinases plus a smaller family of sugar kinases with substrate binding regions in common with homoserine kinases were defined (Bork et al. 1993). Later, a fourth family of receptor kinases (ROK), which also comprises sugar kinases, was added (Titgemeyer et al. 1994). The *E. histolytica* fructokinase belongs to the ribokinase family. In the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/), homologs of the *E. histolytica* fructokinase are present in *Entamoeba nuttalli*, *Entamoeba dispar*, and *Entamoeba invadens*. A BlastP search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the closest relatives outside the genus Entamoeba were from *Prevotella* spp.; one of these gene products was characterized as a fructose 6-kinase (EC 2.7.1.4) (Fuse et al. 2013).

In the present study, we investigated whether *E. histolytica* is able to grow in a medium with fructose replacing glucose and if this medium switch would cause an upregulation of the putative fructokinase gene on the messenger RNA (mRNA) level and on the level of enzyme activity. The *E. histolytica* fructokinase was expressed in *Escherichia coli*, and its substrate specificity and kinetic parameters were measured. Finally, the enzyme was localized by confocal immunofluorescence.

Materials and methods

Microorganisms

E. histolytica trophozoites (HM-1: IMSS) were grown axenically in TYI-S-33 medium (Diamond et al. 1978) containing 10 % (*v*/*v*) bovine serum at 37 °C. The cells were harvested after 48 h of incubation by centrifugation at 500×*g* for 5 min, followed by two washings with phosphate-buffered saline (PBS). For experiments performed with fructose-adapted amoebae, the harvested trophozoites were transferred to medium containing 10 g/l (55.5 mM) fructose, instead of 10 g/l (55.5 mM) glucose. Fructose-adapted cultures remained viable for at least 12 months.

E. coli strain INVαF′ [F′ *end*A1 *rec*A1 *hsd*R17 (r_k^-,m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 λ^-] (Invitrogen, Life Technologies) was used for the direct cloning of the PCR-amplified fructokinase gene. *E. coli* strain BL21-AI [F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm araB*::*T7RNAP-tetA*] from the same provider was used for protein expression.

Cloning and recombinant expression of *E. histolytica* fructokinase

The coding sequence from the *E. histolytica* intronless fructokinase gene (XM_646995) was amplified by PCR from genomic *E. histolytica* DNA which was prepared using the DNeasy Blood and Tissue Kit (Qiagen). The primers 5′-CCG GCT AGC ATG AAC CAT AAA AAA ATT AAA GTA G-3′ and 5′-CAT CCA GCT CGA GTT AGT GAT GGT GAT GGT GAT GTT TTA ACT CAG ATA AAA GCT C-3′ were used for amplification. PCR was performed with Phusion High-Fidelity DNA Polymerase (Thermo Scientific), and the resulting fragment was gel-purified with the QIAquick Gel Extraction Kit (Qiagen) and cloned into the vector pCR II using the TA Cloning Kit Dual Promoter (Invitrogen, Life Technologies). The nucleotide sequence was checked by sequence analysis (Microsynth, Balgach, Switzerland). After digestion with EcoRI, purification was performed with the QIAquick PCR Purification Kit, and the product was ligated into the pET-17b vector (Novagen).

For expression, the plasmid was transformed into BL21-AI competent *E. coli*. Induction was performed at OD_{600}=0.4 with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.2 % (*w*/*v*) arabinose, followed by 4 h culture at 37 °C. Cells were harvested by centrifugation at 5000×*g* for 10 min at 4 °C, resuspended in native lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 100 μg/ml lysozyme, pH 8.0, and disrupted in a mortar. The crude lysate was centrifuged at 18,000×*g* at 4 °C to remove debris, and the recombinant protein in the supernatant with a predicted molecular mass of 33.6 kDa was purified under native conditions using Ni-NTA Spin Columns (Qiagen), and the obtained fractions were analyzed by 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Quantitative reverse transcription PCR (qRT-PCR)

The expression levels of fructokinase mRNA were examined by qRT-PCR of amoebae either grown in normal medium containing glucose or of amoebae adapted to fructose for 4 weeks. Moreover, expression levels of trophozoites freshly switched to fructose medium for a total of 2 or 4 h were investigated.

Total RNA extraction was performed with the GeneJET RNA Purification Kit (Thermo Scientific). The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used for reverse transcription. The reaction using a final total RNA concentration of 25 ng/μl was run at 42 °C for 70 min followed by 6 min inactivation at 70 °C. For dilution series,

5 μl aliquots were diluted 1:10 and used in qRT-PCR, always carried out in duplicate. The master mix consisted of 3.5 mM $MgCl_2$, 1×PCR-buffer B2 (Solis Biodyne), 0.2 mM dNTP mix (Thermo Scientific), 0.8×Eva Green Dye (Biotium), and 1U HOT FIREPol DNA Polymerase (Solis Biodyne). To a final reaction volume of 20 μl, 2 μl template cDNA and 250 nM each (final concentration) of forward and reverse primer were added. For primer design, the program Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used, and the primers were checked for secondary structures (http://mfold.rna.albany.edu/?q=mfold/dna-folding-form) and dimers (http://www.premierbiosoft.com/netprimer/). Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check for pseudogenes or other homologs. The sense and antisense primers were the following: 5′-GGT GAG GTT GTT TGG GAT TG-3′ and 5′-TTC CAA CAG CAA TGA AAG CA-3′. qRT-PCR was carried out with the Roche Light Cycler 480 II using the following protocol: 95 °C for 15 min, 45 cycles of (95 °C, 15 s, 60 °C, 30 s, and 72 °C, 20 s), and a final extension at 72 °C for 10 min. Experiments were performed in triplicate, and positive and negative controls were included in each run. Hexokinase 2 (XM_650873) was used as reference gene, and statistical analysis was performed with the program REST ("relative expression software tool"), available at http://rest.gene-quantification.info/ (Pfaffl et al. 2002).

Kinetic parameters and substrate specificity

The assay for analysis of fructose phosphorylation activity measured the ADP generated in the fructokinase reaction (ADP assay). The decrease of NADH in the coupled lactate dehydrogenase reaction was examined spectrophotometrically at 340 nm. The standard assay mixture (slightly modified from Kroschewski et al. (2000)) contained 1 mM fructose, 2 mM ATP, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM NADH, 0.4 mM phosphoenolpyruvate, 6 U/ml pyruvate kinase (from rabbit muscle, Sigma-Aldrich), 6 U/ml lactate dehydrogenase (from porcine heart, SERVA), and 50 mM Tris–HCl pH 8.0. Ten micrograms of recombinant *E. histolytica* fructokinase was added to a total volume of 1 ml, and the reaction was monitored over a time period of 1 min. To optimize the reaction, various pH (pH 6–9) and temperature conditions (RT, 37 °C) were tested at various fructose concentrations (0.005–10 mM). Measurements with the addition of $MnCl_2$ or $CaCl_2$ (10 mM) and in the absence of $MgCl_2$ were also carried out. Moreover, the putative phosphorylation of glucose, mannose, and galactose (starting concentration: 5 mM) by the recombinant fructokinase was examined. All experiments were carried out in triplicate, and mean values were used for analysis. K_m and V_{max} were calculated with the software GraFit, Version 7 (Erithacus Software Ltd., UK), using the non-linear curve fitting program.

To analyze if the product of the fructokinase reaction was fructose 6-phosphate, the assay was coupled to the phosphoglucose isomerase and glucose-6-phosphate dehydrogenase reactions, and NADPH formation was measured. The assay mixture contained 1 mM fructose, 2 mM ATP, 10 mM $MgCl_2$, 50 mM Tris–HCl pH 8.0, 0.2 mM $NADP^+$, 6 U/ml glucose-6-phosphate dehydrogenase (from baker's yeast, Sigma-Aldrich), 0.1 U/ml phosphoglucose isomerase (from baker's yeast, Sigma-Aldrich), and 10 μg of recombinant fructokinase.

Moreover, fructokinase activity was examined in lysates of *E. histolytica* cells via measurements of NADPH formation, using the same assay mixture as above. The activity was measured in amoebae either grown in fructose or in glucose. For lysate preparation, trophozoites were washed in PBS twice, resuspended in extraction buffer (50 mM Tris–HCl, 1 mM EDTA, pH 7.4), and broken with a Dounce homogenizer. After centrifugation at 14,000×*g* for 5 min, protein concentration of the supernatant was determined with the Bradford assay (Bio-Rad) and 500 μg of total lysate proteins were used per reaction.

Immunofluorescence assay

E. histolytica trophozoites were cultured and fixed in 4-well μ-Slides (ibidi, Martinsried, Germany). Seven hundred microliters of cell suspension was pipetted into the wells, and the slides were incubated at 37 °C for 2 h in a box containing Anaerocult A (Merck). The following steps were all carried out at room temperature. *E. histolytica* cells were fixed with 4 % (*w*/*v*) paraformaldehyde (Sigma-Aldrich) in PBS for 20 min, followed by a washing step with PBS. Afterwards, the cells were incubated for 10 min with 50 mM ammonium chloride (Sigma-Aldrich) and washed with PBS twice. Incubation with 0.1 % (*w*/*v*) saponin (Sigma-Aldrich) in PBS and mouse antiserum (Davids Biotechnologie, Regensburg, Germany) 1:500 was performed for 1 h. As negative control, pre-immune serum was used. After three washings with PBS, amoebae were stained with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Molecular Probes) 1:1000 in PBS for 30 min in the dark. Then, three washing steps followed, and to stain the nuclei, 5 min of incubation with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) 1:2000 in water was performed. After three more washings, cells could be stored in PBS in the dark. Microscopy was carried out with the LSM 700 confocal microscope (Carl Zeiss, Germany), and pictures were analyzed with ZEISS Efficient Navigation (ZEN) imaging software.

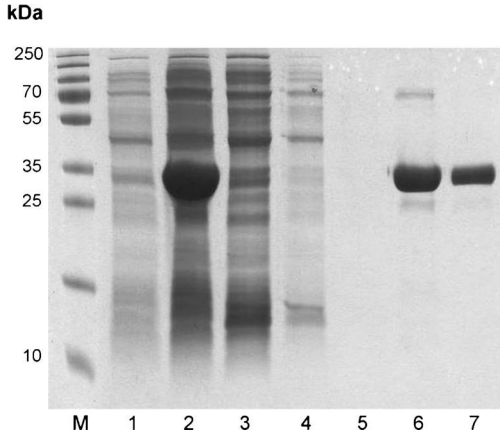

Fig. 1 SDS-PAGE showing the purification of recombinant *E. histolytica* fructokinase. *Lane 1 E. coli* lysate from non-induced cells, *lane 2* lysate from arabinose and IPTG-induced cells, *lane 3* flow-through fraction after binding of proteins, *lanes 4–5* washing fractions, *lanes 6–7* elution fractions containing the purified recombinant fructokinase. Marker proteins are shown on the left side (M)

Results

E. histolytica trophozoites can be cultured in medium containing fructose

For all experiments with fructose-adapted amoebae, cells were grown in medium containing 10 g/l fructose instead of 10 g/l glucose for 4 weeks. Until now, after more than 12 months, the amoebae continue to proliferate well in the fructose medium.

Cloning and recombinant expression of the *E. histolytica* fructokinase

As the *E. histolytica* hexokinases are unable to phosphorylate fructose (Kroschewski et al. 2000), the putative fructokinase was studied. Its open reading frame (XM_646995) was amplified by PCR, and the resulting fragment was engineered into the pET-17b vector. The protein was expressed abundantly (around 15 μg/ml) in *E. coli* BL21-AI cells from which it was purified under native conditions using Ni-NTA Spin Columns. Stored at −20 °C in 50 % (*v*/*v*) glycerol, the enzyme was stable for at least 6 months. SDS-PAGE analysis of the purified protein revealed a band with an apparent molecular mass of slightly below 35 kDa, corresponding to the calculated molecular mass of 33.6 kDa including the hexahistidine tail (Fig. 1).

Upregulation of the fructokinase mRNA in amoebae switched to fructose medium

E. histolytica trophozoites were switched from 10 g/l glucose to 10 g/l fructose medium. Total RNA was extracted from the original culture and after 2 and 4 h in fructose medium. After reverse transcription, the relative expression of fructokinase mRNA was determined by qRT-PCR (Fig. 2). In general, only a modest upregulation of fructokinase expression was observed, 1.47-fold after 2 h ($p<0.05$) and 1.81-fold after 4 h ($p<0.005$). Compared to amoebae grown in glucose medium, the expression of fructokinase mRNA in amoebae grown in fructose medium for 4 weeks remained elevated 1.5-fold ($p<0.05$). The efficiency of amplification was between 0.85 and 1.

Kinetic parameters and substrate specificity of *E. histolytica* fructokinase

E. histolytica fructokinase was produced at an estimated 30 % of the soluble protein in *E. coli* (Fig. 1), and about 1 μg of recombinant protein per microliter of eluate could be purified by nickel chelate affinity chromatography under native conditions. For measurements of fructokinase activity, the ADP generated was measured by coupled pyruvate kinase and lactate dehydrogenase reactions, and the decrease of NADH was examined spectrophotometrically at 340 nm. The activity of

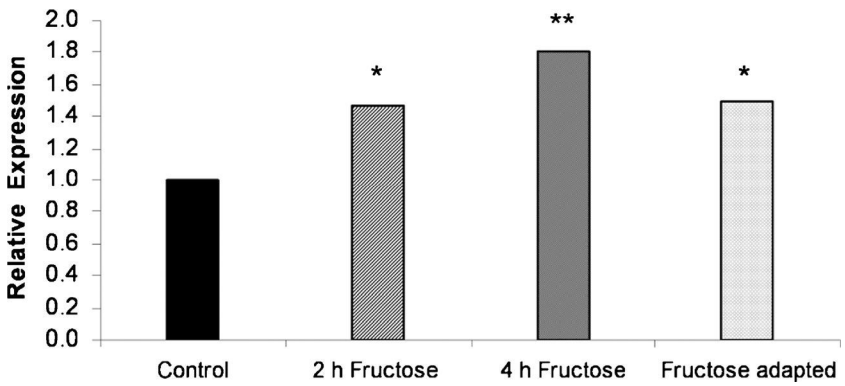

Fig. 2 *E. histolytica* fructokinase mRNA expression of amoebae grown in medium containing fructose. *E. histolytica* trophozoites grown in fructose for 2 h upregulated fructokinase mRNA by the factor 1.47 ($p<0.05$). Highest expression ratios were observed after 4 h of cultivation in fructose medium with a mean upregulation of 1.81 ($p<0.005$). Compared to cells grown in normal medium, fructose-adapted amoebae showed an upregulation of 1.5 ($p<0.05$)

the fructokinase was $MgCl_2$ dependent, with an optimum concentration of 10 mM (used in all experiments); no activity was observed with the addition of $CaCl_2$. A slightly diminished fructokinase activity of 83 % was detected with 10 mM $MnCl_2$ instead of $MgCl_2$. The activity was rising up to a substrate concentration of 1 mM (108.5 U/mg; Fig. 3a, Table 1) and dropping to about half of the maximum at 5 mM substrate concentration. Calculated V_{max} of *E. histolytica* fructokinase at 37 °C was 131.3±8.1 U/mg protein, and K_m for fructose was 0.156±0.032 mM (Fig. 3b). Maximal phosphorylation activity was found at pH 7 (125.4±6.7 U/mg), used in all assays. Lower activity was observed at pH 6 (81.3±6.7 U/mg), pH 8 (116.8±6 U/mg) and pH 9 (108.5±3.3 U/mg) (Fig. 3c).

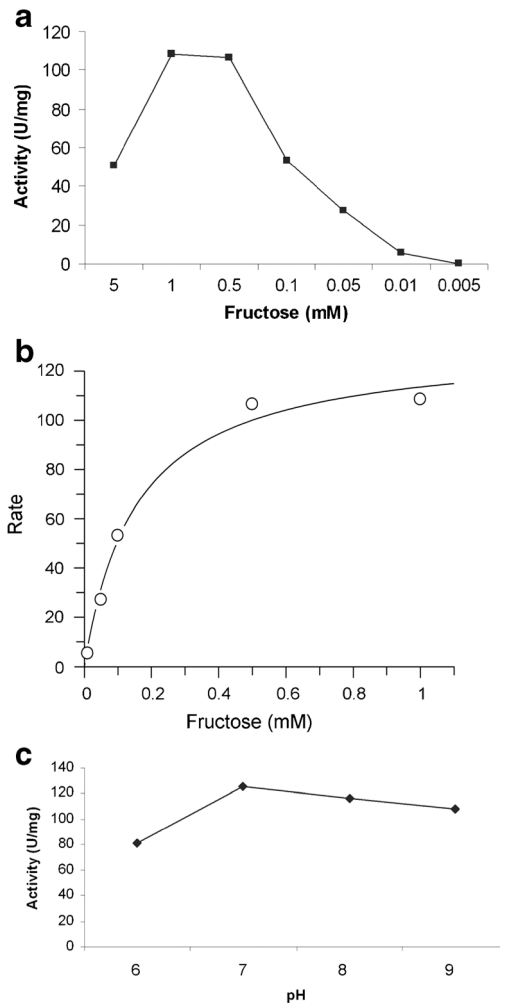

Fig. 3 **a** Fructose phosphorylation activity in the fructokinase reaction with different substrate concentrations. Generated ADP was measured spectrophotometrically via the decrease of NADH in the coupled lactose dehydrogenase reaction, at the temperature optimum of 37 °C. Highest fructokinase phosphorylation activity was observed with 1 mM fructose (108.5 U/mg). Similar results were observed with 0.5 mM fructose, whereas the activity decreased using lower concentrations. At 5 mM fructose concentration, the activity decreased to about half of that observed at 1 mM. **b** Fructokinase reaction rate as a function of fructose concentration (0.01–1 mM). Calculated V_{max} of *E. histolytica* fructokinase at 37 °C was 131.25 U/mg protein and K_m was 0.156 mM. **c** pH dependency of *E. histolytica* fructokinase. Maximal phosphorylation activity was found at pH 7 (125.4±6.7 U/mg) whereas lower activities were observed at pH 6 (81.3±6.7 U/mg), pH 8 (116.8±6 U/mg) and pH 9 (108.5±3.3 U/mg)

Next, the activity of the recombinant enzyme with glucose, mannose, and galactose was tested. Limited activity with mannose but no activity with glucose or galactose was observed (Table 1). Moreover, the temperature optimum of *E. histolytica* fructokinase was at 37 °C (data not shown).

Potentially, the fructokinase reaction can generate fructose 1-phosphate or fructose 6-phosphate. To test for the activity generating fructose 6-phosphate, a coupled assay including glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase was performed and the formation of NADPH during the latter reaction was measured by spectrophotometry. The calculated activity at 1 mM fructose concentration was 26.3±1.1 U/mg protein with a turnover number of 14.3±0.6 molecules per second, demonstrating the 6-phosphate forming activity of *E. histolytica* fructokinase.

Fructokinase activity is elevated in lysates from fructose-adapted *E. histolytica* trophozoites

Fructokinase activity was examined in *E. histolytica* lysates via measurements of NADPH formation. In lysates of amoebae adapted to fructose, the measured fructokinase activity was 3-fold higher than in control amoebae. The calculated activity in fructose-adapted trophozoites was 12.3±1.5 U/mg protein; the turnover number was 6.7±0.8 molecules per

Table 1 Activity of the recombinant *E. histolytica* fructokinase using different substrates at various concentrations

	Activity (μmoles/min/mg)	Turnover (molecules/s)
Fructose (mM)		
5	50.5	27.5
1	108.5	59.1
0.5	106.5	58.0
0.1	53.1	29.0
0.05	27.1	14.8
0.01	5.3	2.9
0.005	0.0	0.0
Mannose (mM)		
5	12.5	6.8
1	2.5	1.4
0.5	0.0	0.0
Galactose (mM)		
5	0.0	0.0
Glucose (mM)		
5	0.0	0.0

second. Control amoebae grown in glucose showed a fructokinase activity of 3.9±1.8 U/mg and a turnover number of 2.1±0.5. So, the level of enzyme activity had risen more than the mRNA level, indicating additional post-transcriptional regulation.

E. histolytica fructokinase localizes to the cytoplasm of trophozoites

Confocal immunofluorescence microscopy was used to test the cellular localization of the fructokinase. A mouse serum was raised against the recombinant enzyme, and antibody binding was visualized with secondary anti-IgG antibodies labeled with Alexa 488 fluorescent dye. For staining of the nucleus, DAPI was used. Cytoplasmic localization was observed in amoebae stained with the fluorescent dye (Fig. 4), controls showed no staining (data not shown).

Discussion

Only limited information exists about the response of *E. histolytica* to a lack of nutrients or changes in nutrient supply. Short-term (12 h) glucose starvation of trophozoites increased target cell lysis; moreover, the virulence of the trophozoites in a hamster liver abscess model was augmented. The lysine-rich protein KRiP1 was found to play an important role in this augmentation of virulence (Tovy et al. 2011). Deprivation of cysteine, a normal medium component, led to drastic changes in the metabolism of trophozoites (Husain et al. 2010). So far, it is not known, however, if this also increases their virulence.

Active glycolysis in *E. histolytica* was associated with uptake of oxygen. This respiration was maximally stimulated by glucose (100 %), much less by galactose (68 %), and even less by fructose (30 %) (Weinbach and Diamond 1974). Nevertheless, in this study, we showed that the trophozoites could be switched from glucose to fructose medium without any problems, and we studied fructokinase as the tool to metabolize fructose.

In the annotated *E. histolytica* genome database, there is a single fructokinase gene. The intronless 885 bp gene codes for a protein of 294 residues with a predicted molecular mass of 32.8 kDa and an isoelectric point of 5.87. Due to its association with bacterial sequences in the phylogenetic tree, the *E. histolytica* fructokinase gene was among the 96 candidates for lateral gene transfer (Loftus et al. 2005) and remained one up to this date with the availability of many more genomes (Grant and Katz 2014). All *Entamoeba* spp. sequenced to this date possess homologs of the fructokinase gene, so the event of lateral gene transfer may have been an early one. Interestingly, the single *E. histolytica* galactokinase gene is also most similar to bacterial galactokinase genes.

The upstream and downstream flanking regions of the *E. histolytica* fructokinase gene were retrieved from the AmoebaDB Database (http://amoebadb.org/amoeba/) and were found to be extremely A/T-rich (83–84 %) and short, only 77 bp to the neighboring upstream gene transcribed from the same strand, and only 64 bp to the downstream gene transcribed from the opposite strand. Whereas, expectedly, no putative signal peptide sequence, transmembrane domain, or glycosylation site were found in the deduced protein sequence; the neural network-based program NetPhos 2.0 (Blom et al. 1999) predicted the amino acid residues Ser128, Ser134, Ser146, Ser169, Ser177, Ser260, Ser273, Thr69, Tyr44, and Tyr220 as possible phosphorylation sites (score >0.8, cutoff >0.5). A high number of predicted phosphorylation sites is quite usual for *E. histolytica* proteins which corresponds to the large number of predicted protein kinases in this organism (Loftus et al. 2005).

In other protist parasites such as the trypanosomatids, *Plasmodium* spp., *Giardia intestinalis*, and *Trichomonas vaginalis*, no fructokinase gene was annotated or purified and characterized biochemically. Of course, this does not exclude such an activity by other sugar kinases. Two older studies on *Leishmania* spp. (Kreutzer and Christensen 1980) and on *Trypanosoma* spp. (Kreutzer and Sousa 1981) report the

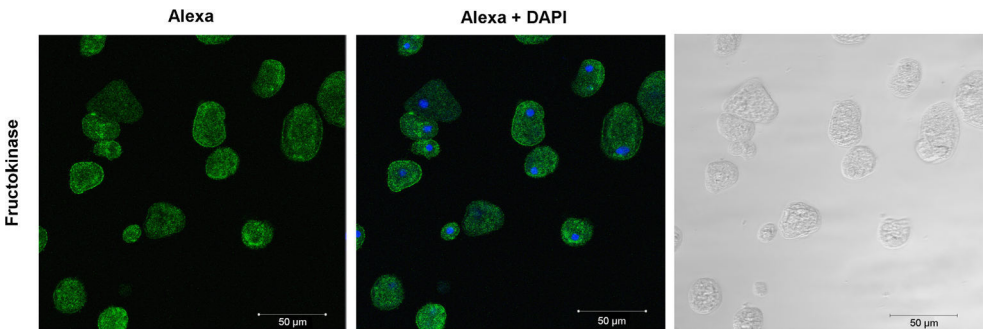

Fig. 4 Localization of *E. histolytica* fructokinase. Paraformaldehyde-fixed trophozoites (*right panel*) were probed with a mouse serum against recombinant *E. histolytica*, and bound antibodies were visualized with Alexa Fluor 488 anti-mouse IgG (*left panel*). Nuclei were stained with DAPI (*middle panel*). No staining was seen when the trophozoites were stained with pre-immune serum (data not shown)

detection of a fructokinase activity by isoenzyme electrophoresis. Mertens and Müller (1990) described a fructokinase in *Tritrichomonas foetus*, as well as a separate glucokinase. The *T. foetus* fructokinase was dependent on ATP, with K_m values of 0.2 mM for fructose and 0.081 mM for ATP.

The *E. histolytica* fructokinase belongs to the large ribokinase family which consists of carbohydrate kinases of various specificities including fructokinases, phosphofructokinases, ribokinases, glucokinases, ketohexokinases such as ketodeoxygluconate kinase, and adenosine kinases (Bork et al. 1993). Several of these (Fuse et al. 2013; Caescu et al. 2004; But et al. 2012; Fennington and Hughes 1996; Perez-Cenci and Salerno 2014; Qu et al. 2004) are listed in Table 2, together with the *E. histolytica* fructokinase and few more bacterial fructokinases of the ROC type (Titgemeyer et al. 1994; Nocek et al. 2011; Thompson et al. 1991; Sato et al. 1993; Scopes et al. 1985). A sequence comparison of the mentioned ribokinase-like fructokinases is shown in Fig. S1. On one hand, similarities are obvious, especially in some fully conserved regions; on the other hand, significant divergence exists. As an example, the *E. histolytica* and *Prevotella intermedia* (Fuse et al. 2013) sequences are 46.8 % identical on the amino acid level.

Table 2 shows some more similarities between the related fructokinases from *E. histolytica* and various bacteria. For instance, the *E. histolytica* enzyme also displayed a limited activity with mannose besides the major fructokinase activity (Table 1). Mannose-phosporylating activity was also observed in the ROC-type fructokinases from *Lactococcus lactis* (Thompson et al. 1991) and *Streptococcus mutans* (Sato et al. 1993), and a trace activity was found in *Zymomonas mobilis* (Scopes et al. 1985). The maximum fructokinase activity of the *E. histolytica* enzyme was observed at 1 mM fructose and decreased significantly at 5 mM substrate concentration (Fig. 3a). Such substrate inhibition was also observed in the fructokinase from potatoes (Renz and Stitt 1993). *E. histolytica* fructokinases and all the bacterial fructokinases mentioned in Table 2 required bivalent ions for their activity, preferentially Mg^{2+} with the exception of *Methylmicrobium alcaliphilum* fructokinase which required Mn^{2+} for its activity (But et al. 2012). In total, the V_{max} of *E. histolytica* was about average compared to the bacterial enzymes, but the K_m was lowest, allowing activity at lower fructose concentrations. Moreover, most species showed similar pH optima as found for *E. histolytica*; only the *M. alcaliphilum* enzyme (But et al. 2012) had a basic pH optimum. The temperature optimum of the *E. histolytica* fructokinase corresponded to the temperature of the human host; the enzyme with the highest activity from *Thermococcus litoralis* (Qu et al. 2004) had the highest optimum temperature of 80 °C.

Recombinant *E. histolytica* fructokinase produced fructose 6-phosphate. On one hand, this is a direct intermediate of classical glycolysis and can be used for the generation of energy. On the other hand, we noted that fructose 6-phosphate can be converted in one step to glucosamine 6-phosphate by a

Table 2 Comparison of bacterial fructokinases to the *E. histolytica* enzyme

Organisms	Kinase family	V_{max} (U/mg)	K_m (mM)	Substrate specifity	Cations	pH optimum	Temperature optimum (°C)	Reference
Entamoeba histolytica	Ribokinase	131	0.156	Fructose, mannose	$Mg^{2+}>Mn^{2+}$	7–8	37	This work
Bifidobacterium longum	Ribokinase	0.84	0.739	Fructose	?	6	50	Caescu et al. 2004
Methylomicrobium alcaliphilum	Ribokinase	141	0.26	Fructose	Mn^{2+}	9	60	But et al. 2012
Prevotella intermedia	Ribokinase	?	?	Fructose	Mg^{2+}	?	?	Fuse et al. 2013
Rhizobium leguminosarum	Ribokinase	31	0.31	Fructose	$Co^{2+}>Mg^{2+}>Cd^{2+}>Mn^{2+}>Ca^{2+}$	8	25	Fennington and Hughes 1996
Synechococcus sp.	Ribokinase	?	?	Fructose, maltose (trace)	Mg^{2+}	7.5	30	Perez-Cenci and Salerno 2014
Thermococcus litoralis	Ribokinase	730	2.3	Fructose	$Mg^{2+}>Mn^{2+}>Co^{2+}$	7.5–8	80	Qu et al. 2004
Bacillus subtilis	ROK	178	0.38	Fructose	?	8	25	Nocek et al. 2011
Lactococcus lactis	ROK	190–200	0.31	Fructose, mannose	$Mg^{2+}>Co^{2+}>Fe^{2+}>Mn^{2+}>Ni^{2+}>Zn^{2+}>Cd^{2+}$	7–8	25–40	Thompson et al. 1991
Streptococcus mutans	ROK	20	0.77	Fructose, mannose	Mg^{2+}	7.4	25	Sato et al. 1993
Zymomonas mobilis	ROK	350	0.7	Fructose, mannose (trace)	?	8	25	Scopes et al. 1985

glucosamine–fructose-6-phosphate aminotransferase (candidate XP_650078) followed by acetylation by a glucosamine-6-phosphate *N*-acetyltransferase (candidates XP_655194, XP_649522, and XP_648703). The resulting *N*-acetylglucosamine 6-phosphate can serve as a direct precursor of chitin in the *E. histolytica* cyst wall.

The mRNA encoding this enzyme was significantly upregulated in amoebae adapted to fructose, as well as in trophozoites cultured with fructose for 2 and 4 h, respectively (Fig. 2). However, the highest fructokinase mRNA upregulation, which was observed after 4 h, was only as high as about 2-fold compared to amoebae grown in normal medium. Thus, the parasite responded to chemical stress only with rather moderate changes of mRNA expression. The fructokinase activity measured in extracts from fructose-adapted *E. histolytica* was about 3-fold higher than in control extracts, pointing to some post-transcriptional regulation. Interestingly, the effect of fructose on the fructokinase expression in *E. histolytica* was comparable to what was found in bacteria before. So, in *Zymomonas mobilis*, the mRNA level increase was 3-fold whereas the fructokinase activity increased about 2-fold when the bacteria were grown on fructose instead of glucose (Zembrzuski et al. 1992).

This work represents the first biochemical study on the *E. histolytica* fructokinase. The enzyme allows the trophozoites to grow on fructose which may be more abundant in the human colon than glucose. In vitro, *E. histolytica* adapted to fructose media without any problems and modestly upregulated fructokinase expression on the levels of mRNA and enzyme activity. So, taken together, the *E. histolytica* fructokinase is a new example for an important metabolic activity, for which the gene was most likely acquired by lateral gene transfer.

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8. *Entamoeba histolytica*: Molecular Characterisation of a DNase Homologous to Bacterial TatD

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Short Title:
TatD nuclease in *Entamoeba histolytica*

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8.1 Abstract

Entamoeba histolytica is the causative agent of invasive amoebiasis associated with dysentery or liver abscess. The nitroimidazole drug metronidazole has been used successfully to treat amoebiasis for almost 50 years, without any significant resistance problems. It is well-established that metronidazole has to be chemically reduced to become toxic to the amoebae, but much less is known about the molecular targets of the active metabolites. One process, which is addressed in this study, is the degradation of chromosomal DNA. When amoebae were treated with 50 micromolar metronidazole for 12 hours or longer, the TUNEL (terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling) DNA damage assay turned positive. One hypothesis suggests reduced metabolites of metronidazole, such as the nitroradical anion, as the DNA damaging agents, whereas the alternative hypothesis postulates an apoptosis-like process involving cellular DNases. The first hypothesis is challenged by the low reactivity of the nitroradical anion as well as by the fact that DNA degradation is also observed when amoebae are treated with non-radical forming agents such as G418. The alternative hypothesis suffers from the absence of many apoptosis-associated endonucleases like caspase-dependent DNase, DNase II or endonuclease G. However, *E. histolytica* possesses a homologue of the bacterial TatD nuclease, for which a role related to apoptosis was described in trypanosomes, leishmania and yeast. We expressed *E. histolytica* TatD in *E. coli* and found a Mg^{2+}-dependent DNase activity of the recombinant protein. Confocal immunofluorescence revealed a cytoplasmic distribution of TatD. Unexpectedly, expression of the mRNA encoding TatD was down-regulated in metronidazole-treated amoebae. The mRNA expression of other putative endonuclease genes not implicated in apoptosis-like cell death, however, showed a modest up-regulation. Our results suggest that DNA destruction is a late event in metronidazole activity against *E. histolytica* and that TatD nuclease could contribute to this process.

8.2 Authors Summary

Entamoeba histolytica is the causative agent of invasive amoebiasis associated with dysentery or liver abscess. For treatment, metronidazole has been used successfully for almost 50 years, without any significant resistance problems. Within *E. histolytica*, the compound is chemically reduced to become active. However, further processes resulting in the death of the amoebae are not understood well. An important example is the degradation of chromosomal DNA, which is observed during the treatment with metronidazole or the compound G418 in a process resembling apoptosis. We hypothesised that apoptosis-related DNases could be involved, but the only candidate we found in the *E. histolytica* genome is a homologue of the bacterial TatD nuclease, for which a role related to apoptosis was described in trypanosomes, leishmania and yeast. Therefore, we expressed *E. histolytica* TatD in *E. coli* and found a Mg^{2+}-dependent DNase activity of the recombinant protein. Confocal immunofluorescence showed cytoplasmic staining of TatD. Unexpectedly, the expression of the mRNA coding for TatD was down-regulated in metronidazole-treated amoebae. Other putative endonuclease genes not implicated in apoptosis-like cell death showed a modest up-regulation. TatD nuclease remains a candidate for the observed DNA degradation, but as in higher organisms, this process most likely will involve further factors.

8.3 Introduction

The protozoan parasite *Entamoeba histolytica* is the cause of amoebic dysentery and liver abscess and affects millions of people worldwide. Between 36 and 50 million cases of disease and up to 110,000 deaths every year were estimated in an older study [1], however, the situation improved in the recent decades [2]. The nitroimidazole drug metronidazole has been used to treat *E. histolytica* infections since 1966 [3] and remains the gold standard drug until now. Unlike for *Trichomonas vaginalis*, *Giardia intestinalis* or bacterial pathogens, no metronidazole-resistant *E. histolytica* strains have emerged, and only partial resistance has been achieved in the laboratory [4].

The mechanisms of action of this drug are still not fully understood, but it is well-established that the reduction of metronidazole is necessary for its activity. It proceeds through nitroradical anions to nitrosoimidazole, a hydroxylamine derivative and to further reduced metabolites [5,6]. A previous study demonstrated that several proteins including thioredoxin reductase and thioredoxin were covalently modified by the metronidazole metabolites within two hours of treatment. As a consequence, the disulfide-reducing activity of the thioredoxin system was diminished [7]. The thioredoxin system plays an important role in the repair of oxidatively damaged proteins, *E. histolytica* thioredoxin is able to interact with more than 200 target proteins [8].

Metronidazole has long been suspected to be the causative agent of DNA damage. *In vitro,* it results in a positive Ames test [9] but initial concerns about teratogenicity could not be substantiated [10]. There is an ongoing discussion about the possible carcinogenicity of metronidazole in humans. An earlier study found no association with cancer [11], however, a recent report including a large number of treated individuals showed some supportive evidence for a weak carcinogenicity of metronidazole [12]. Whereas in the human host, DNA damage may be very limited and is repairable, in metronidazole-treated parasites, massive DNA degradation is observed. So far, it is not known how exactly DNA is degraded in the parasite. One hypothesis, based on the interaction of electrolytically reduced metronidazole with *Escherichia coli* or phage DNA, suggests that DNA degradation in metronidazole-treated cells may be

caused by direct action of the nitroradical anions [13]. However, this intermediate is not highly reactive and doubts about its ability to break DNA strands have been raised [14]. A further reduced metabolite was shown to react with guanosin bases [15] but this does not necessarily lead to strand cleavage.

Research on apoptosis during the last two decades raised the alternative hypothesis that DNA damage might be elicited by enzymes in the parasite. In early years of apoptosis research, this process was seen as a way of multicellular organisms to eliminate unnecessary cells. However, processes resembling apoptotic cell death, always including the degradation of chromosomal DNA, were also observed in single cell organisms such as *Trypanosoma cruzi* [16], *Leishmania amazonensis* [17], *Saccharomyces cerevisiae* [18] and others. Several in-depth reviews deal with the evolutionary implications of these findings [19,20]. Recent reports show similar observations in *E. histolytica*. Oxidative stress elicited by hydrogen peroxide [21,22] or nitric oxides [23] as well as G418 [24] and also metronidazole [K. Seifert, unpublished data] led to signs of apoptotic cell death. These include exposure of phosphatidylserine on the outer membrane leading to Annexin V binding, and DNA degradation as determined electrophoretically or by TUNEL (terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling) assays [25]. Apoptosis-like DNA degradation after metronidazole treatment has also been reported in *Blastocystis hominis* [26] and in *Giardia intestinalis* [27,28], where the authors concluded that an autophagy type of programmed cell death could have occurred [28].

In multicellular organisms, several delicately controlled pathways to apoptosis exist. Central signals in mammals, insects and worms are transferred by a cascade of caspases (aspartate-specific cysteine proteases) [29]. Such caspases are absent in plants, fungi, protozoans and prokaryotes, however, two related protein families are found, paracaspases and metacaspases. The latter are present in protozoans, plants, fungi and bacteria whereas paracaspases were found in metazoans and *Dictyostelium* only [30]. Among the protozoan parasites, metacaspases were found in *Plasmodium*, *Trypanosoma*, *Leishmania*, *Acanthamoeba* and *Trichomonas*, but surprisingly not in *E. histolytica* and *Giardia*. Taken together, this part of the cell death machinery appears to be more complex in higher organisms.

As is the case for apoptotic signalling, the apoptotic DNase system is also more complex in higher organisms [31]. The major endonuclease players are DFF40/CAD (40 kDa DNA fragmentation factor / caspase-activated DNase), the acidic DNase II responsible for DNA degradation in endocytotic vesicles, the mitochondrial endonuclease G, the ubiquitous FEN-1 (flap endonuclease-1) and TatD DNase. In *E. histolytica*, DFF40/CAD, DNase II and endonuclease G are absent, only TatD and FEN-1 are found encoded in the genome. DFF40/CAD is reserved to higher organisms. In unicellular organisms, *S. cerevisiae* has all four remaining DNases, *Trypanosoma* and *Leishmania* possess endonuclease G, TatD and FEN-1, whereas in *Giardia* and *Trichomonas,* DNase II, TatD and FEN-1 are found.

FEN-1 is a special nuclease linked to apoptotic cell death with its normal function being to cut flaps, which are short single-stranded DNA overhangs, during DNA replication and repair [32]. A FEN-1 homologue is also present in *E. histolytica*, but as it is not believed to cut intact DNA, we did not include FEN-1 in our studies at this time.

E. histolytica had the minimal number of apoptotic DNases with TatD being the only one expected to cut intact DNA. TatD was discovered in *E. coli* as an open reading frame in the *tat* (twin arginine transport) operon. The Mg^{2+}-dependent DNase activity of the TatD protein was first described by Wexler and colleagues in 2000 [33], but soon after, it became clear that TatD homologues are ubiquitous in prokaryotes and eukaryotes. In *Caenorhabditis elegans,* an RNAi screen showed that the TatD nuclease (termed CRN-2, cell death related nuclease-2) represents an apoptotic nuclease [34]. The authors reported that this complex process involves multiple nucleases and non-nuclease cofactors, for example CPS-6, an endonuclease G homologue, or NUC-1, which encodes a type II DNase. In addition, seven other cell death related nucleases (CRNs) including the TatD homologue were found. It is suggested that at least two independent pathways exist which participate in apoptosis and DNA degradation [34].

In *S. cerevisiae*, apoptosis-like cell death is observed upon treatment with hydrogen peroxide [35]. This organism has an intermediate number of cell death related nucleases, fewer than the multicellular organisms, but retaining endonuclease G and DNase II which are missing in *E. histolytica*. Knockout of

yeast TatD decreased the sensitivity to H_2O_2 whereas TatD overexpression resulted in an increase of sensitivity to oxidative stress [35].

The topoisomerase IB inhibitor baicalein led to apoptotic cell death in *Leishmania donovani* [36], which was independent of caspase-like proteases or metacaspases present in this parasite. *L. donovani* endonuclease G was found to interact both with TatD nuclease and also with FEN-1 nuclease, while TatD and FEN-1 did not interact. The authors concluded that TatD, FEN-1 and endonuclease G form a DNA degradesome which is responsible for caspase-independent DNA degradation.

Treatment of the bloodstream form of *Trypanosoma brucei* with prostaglandin D2 (PGD2) leads to growth inhibition and apoptosis-like death of the parasites [37]. As in *L. donovani*, complex formation between TatD nuclease and endonuclease G was shown, here by co-immunoprecipitation [38]. When the expression of TatD was down-regulated by RNAi, the effect of PGD2 on the bloodstream forms or of H_2O_2 on the procyclic forms was diminished.

In this study, we cloned and expressed the *E. histolytica* TatD homologue in *E. coli*, examined its DNase activity and localised the protein in the cytoplasm of *E. histolytica*. Metronidazole, surprisingly, led to a down-regulation of the mRNA encoding TatD. We also performed a search for other *E. histolytica* endonucleases which had not been described as linked to apoptosis. We examined their expression under the influence of metronidazole and found two repair endonucleases and a LINE (long interspersed element) endonuclease being up-regulated.

8.4 Materials and Methods

Culture of parasites

E. histolytica trophozoites (strain HM-1:IMSS) were cultured in TYI-S-33 medium containing 10% (v/v) bovine serum [39] at 37°C, and experiments were performed 48 h after splitting of the cultures.

Preparation of *E. histolytica* lysate

Trophozoites were harvested after 48 h of incubation by centrifugation at 500 x *g* for 5 min and washed with PBS twice. The cells were then lysed by vortexing in PBS containing 0.05% (v/v) Nonidet P-40 (Sigma-Aldrich), followed by centrifugation at 14,000 x *g* for 5 min. The supernatant was used for experiments on the same day. Lysate protein concentration was determined using the Bradford assay (Bio-Rad).

Nuclease assay

For the determination of DNase activity, pUC19 plasmid DNA [40] (available at Fermentas, now Thermo Scientific), propagated in *E. coli* XL-1 Blue, was used as substrate. Supercoiled or linearised plasmid DNA (1 µg), respectively, was incubated with 10 µg *E. histolytica* cell lysate protein for 10 min, 30 min or 60 min, in a volume of 20 µl in PBS with or without 10 mM $MgCl_2$. Alternatively, pUC19 DNA was incubated with 1 µg of recombinant *E. histolytica* TatD nuclease (see below) for 1 h, at 37°C in 50 mM Tris-HCl, pH 7.0. Samples were analysed by electrophoresis on 1% agarose gels with ethidium bromide staining followed by UV illumination to visualize DNA. Enzyme activities of recombinant TatD were tested with the addition of different metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and Fe^{3+}) at various concentrations. Preliminary experiments at pH values of the assay buffer between pH 5.0 and pH 9.0 had revealed only slight differences (data not shown), therefore all experiments were performed at pH 7.0.

TUNEL assay of metronidazole-treated *E. histolytica* trophozoites

TUNEL (terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling) visualizes DNA strand breaks by labeling of the exposed 3´-OH groups followed by fluorescence detection [25]. The TUNEL assay was carried out with the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Germany) according to the manufacturer's protocol. Briefly, trophozoites were treated at a final concentration of 50 µM metronidazole (Sigma-Aldrich) for time periods ranging from 0 h to 24 h with intervals of 3 h in comparison to untreated control cells. Two x 10^6 metronidazole-treated or control cells were fixed with paraformaldehyde solution (4% (w/v) in PBS, pH 7.4) at room temperature for 30 min. The cells were washed twice with PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 5 min on ice, followed by two washes with PBS. The TUNEL reaction mixture (containing label and enzyme solution) was added and the amoebae were incubated for 60 min at 37°C in a humidified chamber in the dark. After washing, the cells were embedded in VECTASHIELD mounting medium containing DAPI (4',6-diamidino-2-phenylindole) nuclear stain (Vector Laboratories, USA) and analysed under an Eclipse E800 fluorescence microscope (Nikon). A negative control (no enzyme solution) and a positive control (DNase I) were included in each experimental set up. All tests were carried out in triplicate.

Database search for DNases

We searched the NCBI (www.ncbi.nlm.nih.gov/protein/) and AmoebaDB (www.amoebadb.org) databases for putative DNase, deoxyribonuclease or nuclease genes from the *E. histolytica* HM-1:IMSS genome, excluding known RNases. In total, 38 unique entries were found (Supplementary Table 1). We focused our study on predicted endonucleases, grouped them into families, and selected a list of 16 putative nuclease genes, including TatD, for qRT-PCR.

Cloning and purification of TatD

E. histolytica genomic DNA was prepared using the DNeasy Blood & Tissue Kit (Qiagen). The coding sequence from the intronless *E. histolytica* TatD gene (XM_646378 in the NCBI database) was amplified by PCR from this DNA using the primers 5'- CGT ACG CAT ATG GCA CAA CAA TTT ATT G -3' and 5'- CAT CCA GCT CGA GTC AGT GAT GGT GAT GGT GAT GAT TCA TAG TTG GGA AAT ACA TG -3'. PCR was performed with Phusion High-Fidelity DNA Polymerase according to the instructions of the supplier (Thermo Scientific). The amplification product was purified using the QIAquick Gel Extraction Kit (Qiagen), digested with the enzymes NdeI and XhoI, cloned into the pET-17b vector (Novagen, Merck Millipore, Germany), and checked by sequence analysis (Microsynth, Balgach, Switzerland). For expression, the plasmid was transformed into *E. coli* BL21-AI (Invitrogen, Life Technologies) and grown to an OD_{600} of 0.4. The production of recombinant TatD was then induced by addition of 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and 0.2% (w/v) arabinose and culture for 4 h at 37°C. Then the cells were harvested by centrifugation and the recombinant protein was purified under native conditions using Ni-NTA Spin Columns according to the instructions of the supplier (Qiagen).

In gel nuclease activity assay

This assay for activity staining of nucleolytic enzymes was performed with slight modifications of the protocol described by Blank et al. [41]. Recombinant TatD or bovine DNase were electrophoresed on a 12.5% native polyacrylamide gel containing 100 µg ml^{-1} salmon sperm DNA and 50 µg ml^{-1} bovine serum albumin (Sigma). After electrophoresis, gels were washed twice for a total of 60 min with 25% isopropanol (v/v) in 10 mM Tris-HCl, pH 7.4. After 2 additional washings with 10 mM Tris-HCl, pH 7.4 for 30 min, gels were incubated overnight at 37°C in 100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. Another washing step with 10 mM Tris-HCl, pH 7.4 for 10 min was followed by 10 min incubation in staining solution (10 mM Tris-HCl, pH 7.4, 0.02% (w/v) toluidine blue). Destaining was performed with 10 mM Tris-HCl, pH 7.4 for 60 min with

several changes of buffer solution. This experiment was also performed with various samples of *E. histolytica* lysate (30 µg protein per lane).

Western blot analysis

E. histolytica trophozoites were washed twice in PBS, resuspended in extraction buffer (100 mM MOPS, 5 mM ascorbic acid, 2 mM DTT, 5 mM $MgCl_2$, pH 6.8) and lysed with a Dounce homogeniser. TatD antiserum was produced in rabbit and mouse according to the manufacturer's protocols (Davids Biotechnologie, Regensburg, Germany). For immunoblots, 30 µg lysate proteins were separated by 12.5% SDS-polyacrylamide gels and transferred onto a Protran nitrocellulose membrane (Whatman, Biometra, Goettingen, Germany) at 300 mA for 1 h at 4°C, using a wet electrophoretic transfer chamber (Hoefer Inc., USA). One h blocking with Blotto (5% w/v nonfat dry milk in TBS, 0.02% (w/v) sodium azide) was followed by washing steps in TBS and 1 h incubation with TatD antiserum, diluted 1:500 in Blotto. After further washings, the membrane was incubated overnight, at 4°C, with alkaline phosphatase labeled anti-rabbit or anti-mouse IgG (Sigma-Aldrich), diluted 1:30,000 in Blotto. Detection was performed with 0.05% (w/v) NBT and 0.05% (w/v) BCIP in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5). Recombinant TatD was used as positive control.

Immunofluorescence assay

For confocal microscopy, metronidazole treated or untreated *E. histolytica* cells were cultured and fixed in 4-well µ-Slides (ibidi, Martinsried, Germany). Cell suspensions (700 µl per well) were pipetted into the wells and the slides were incubated at 37°C in a box containing Anaerocult A stripes (Merck, Germany) for at least 2 h to achieve a confluent layer of trophozoites under anaerobic conditions. Experiments were performed with amoebae treated with 50 µM metronidazole for 2 h, 4 h, 6 h and 12 h, respectively. Cells were fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) in PBS for 20 min, followed by a washing step with PBS. Fixed cells can be stored in PBS at 4°C. Then the cells were incubated for 10 min with 50 mM ammonium chloride, followed by two washings with PBS. Incubation with 0.1% saponin (w/v) (Sigma-

Aldrich) in PBS and anti-TatD mouse antiserum (Davids Biotechnologie, Regensburg, Germany) 1:200 was performed for 1 h at room temperature. Pre-immune serum was used as negative control. After 3 washings with PBS, amoebae were stained with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, now Life Technologies) 1:1000 in PBS for 30 min in the dark. After three washing steps, 5 min of incubation with DAPI (Sigma-Aldrich) 1:2000 in H_2O followed and after three more washings, cells can be stored in PBS in the dark. Confocal laser scanning microscopy was performed with the LSM 700 confocal microscope (Carl Zeiss, Germany) and pictures were analysed with ZEN (ZEISS Efficient Navigation) imaging software.

Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed to examine the basal mRNA expression levels of 16 putative *E. histolytica* nuclease genes, including TatD (Supplementary Table S1), in comparison to expression levels after metronidazole treatment. Trophozoites were incubated at 37°C with 50 µM metronidazole, for 1 h and 4 h, respectively. Moreover, TatD mRNA expression levels were also investigated after 8 h and 12 h of metronidazole treatment. Total RNA extraction was carried out with the Gene JET RNA Purification Kit (Thermo Scientific) according to the manufacturer's protocol. For the reverse transcription of mRNA to cDNA the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used. Total RNA had a final concentration of 25 ng/µl. The cDNA synthesis mix was run with a synthesis step at 42°C for 70 min and inactivation at 70°C for 6 min. Five µl aliquots were diluted 1:10 and used for dilution series in RTq-PCR, always pipetted in duplicate. Two µl template was added to the master mix which consisted of 3.5 mM $MgCl_2$, 1 x PCR-buffer B2 (Solis Biodyne), 0.2 mM dNTP mix (Fermentas), 0.8 x EvaGreen Dye (Biotium), 1U HOT FIREPol DNA Polymerase (Solis Biodyne) and 250 nM each of forward and reverse primer, in a final reaction volume of 20 µl. Primers were designed with the program Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and checked for secondary structures (http://mfold.rna.albany.edu/?q=mfold/dna-folding-form) and dimers (http://www.premierbiosoft.com/netprimer/). Finally, Primer-BLAST

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check for pseudogenes or other homologs. The oligonucleotides selected for qRT-PCR are shown in Supplementary Table S1. qRT-PCR was carried out with the Roche Light Cycler 480 II using the hexokinase 2 gene (XM_650873) for reference. The hexokinase 2 primers were forward 5´- TTG AAG CAC TTG ACC TTT TAC AAC C -3´ and reverse 5´- CAG CAC CAA GTC CAG ATC CAT -3´. The run protocol was as follows: 95°C for 15 min, 45 cycles of (95°C, 15 s; 60°C, 30 s and 72°C, 20 s) and a final extension at 72°C for 10 min. Positive and negative controls were included in each run. Experiments were performed in triplicate and for statistical analysis the program REST ("relative expression software tool") was used. The tool was developed by Pfaffl and colleagues [42] and is found at http://rest.gene-quantification.info/. This relative calculation procedure is based on the mean C_p of experimental groups.

8.5 Results

Lysate from *E. histolytica* trophozoites contains endo-DNase activity

Incubation of the closed circular form of pUC19 plasmid with *E. histolytica* cell lysate revealed nuclease activity in the lysate resulting in nicked open circular and linear forms of the plasmid (Figure 1). Longer incubation times and addition of $MgCl_2$ increased the activity. After 30 min incubation with 10 mM $MgCl_2$, no supercoiled plasmid DNA was visible any more, only the linearised and the open circular forms were observed.

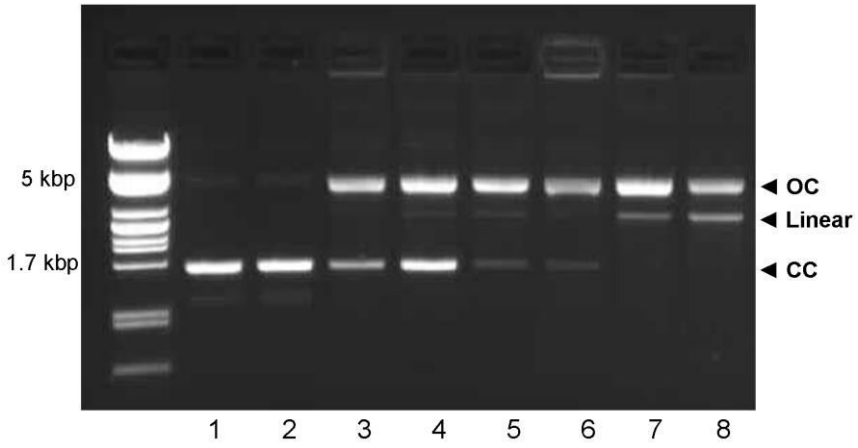

Figure 1. Endonucleolytic digestion of the plasmid pUC19 DNA by *E. histolytica* lysate. Lanes 3–5, pUC19 DNA closed circular form (CC) incubated for 10 min, 30 min or 60 min with *E. histolytica* lysate (no $MgCl_2$ added) was cleaved to the open circular form (OC) and a minor amount of linearised form (Linear). Lanes 6–8, during same incubation times with addition of 10 mM $MgCl_2$, the CC form disappeared after 30 min and an increasing portion of linearised plasmid appeared. Lanes 1 and 2, controls of plasmid DNA alone and plasmid DNA incubated with $MgCl_2$ showed no changes.

Cleavage of chromosomal DNA occurs in metronidazole-treated amoebae

E. histolytica trophozoites were treated with 50 µM of metronidazole and TUNEL assays were performed after several time points. Fluorescence microscopy revealed TUNEL positive trophozoites after 12 h of 50 µM metronidazole treatment (Figure 2), no positive results were observed with shorter incubation times of 3 h, 6 h and 9 h. Longer incubation times resulted in a growing number of TUNEL positive amoebae and nuclei appeared with a brighter fluorescence. Moreover, rounding and shrinkage of cells increased in time. Untreated control cells showed no DNA fragmentation.

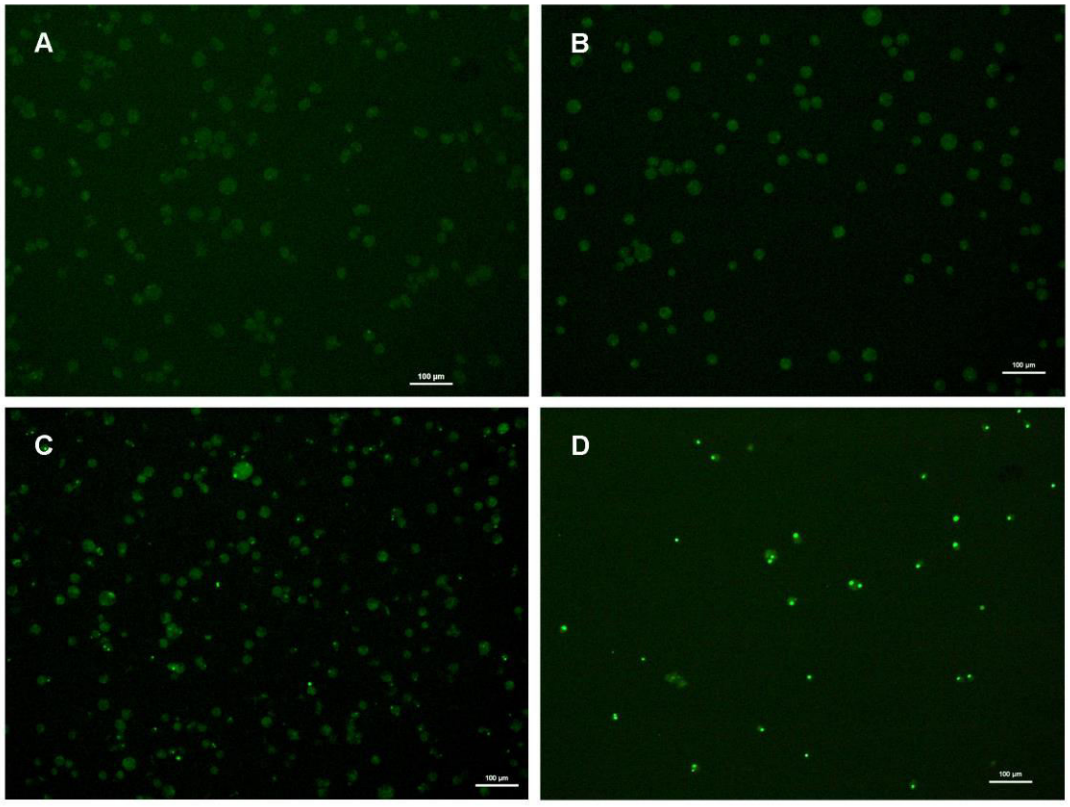

Figure 2. TUNEL assay showed DNA fragmentation in metronidazole treated *E. histolytica* trophozoites. TUNEL positive cells were observed after 12 h of 50 µM metronidazole treatment (C). After 24 h incubation time, TUNEL positive cells increased and showed a clear staining of the nuclei (D). Only background staining was found in control amoebae (A) and after 6 h of metronidazole treatment (B). Bars in images represent 100 µm.

Sequence analysis

As described above, caspase-activated DNase, DNase II and endonuclease G are missing in *E. histolytica*. Therefore, the homologue of bacterial TatD nuclease is the remaining endonuclease candidate for being involved in apoptosis-like cell death. The *T. brucei* TatD (XP_828684) [38], was used to search the *E. histolytica* database by BlastP. The closest hit XP_651470 is annotated as “hydrolase TatD family protein“, and represents a protein of 35.0 kDa and a predicted pI of 6.1. Both proteins share 33% amino acid sequence identity. The BlastP search with *T. brucei* TatD also revealed two other truncated entries in *E. histolytica* (XP_648592 and XP_656369) which were more distantly related and were therefore not characterised any further.

The TatD sequences of all five *E. histolytica* strains are identical, the homologues from *E. dispar* and *E. invadens* share 96% and 62% protein sequence identity with *E. histolytica* TatD, respectively. Supplementary Figure S1 shows the protein sequence alignment of the *E. histolytica*, *T. brucei*, *L. donovani*, *S. cerevisiae*, *C. elegans*, *E. coli* and human TatD homologues generated by ClustalW [43]. The *E. histolytica* sequence displays 30% identity to *E. coli* TatD, for which the DNase activity has been described first [33], and surprisingly, as much as 40% identity to human TatD. The residues important for enzyme activity His151, His176 and Asp224 [38], numbered according to the *E. histolytica* sequence, are conserved in all seven sequences. Interestingly, there is an unpublished structure of *E. histolytica* TatD deposited by Edwards et al. into the NCBI Database (MMDB ID: 76509), in which the three conserved residues are found in close vicinity in a cleft, most likely forming the active site.

Cloning and recombinant expression of the *E. histolytica* TatD endonuclease

The intronless TatD gene including the sequence for a carboxy-terminal hexahistidine tail was amplified from *E. histolytica* genomic DNA and cloned into pET-17b. The recombinant protein was expressed from *E. coli* BL21-AI and purified by metal chelate chromatography under native conditions (Figure

3). From the lysate containing a massive amount of recombinant TatD, sufficient soluble protein could be prepared.

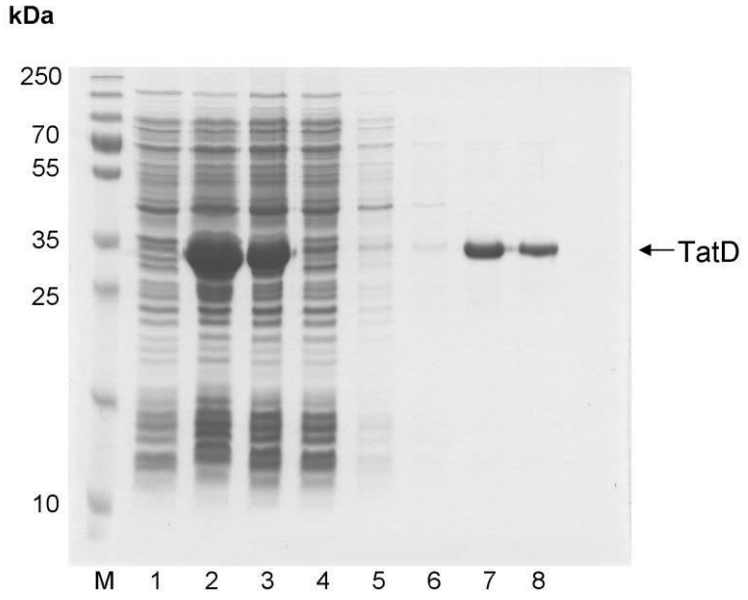

Figure 3. SDS-PAGE showing the purification of recombinant TatD. 1, *E. coli* lysate from non-induced cells; 2, lysate from arabinose and IPTG-induced cells; 3, cleared lysate; 4–6, washing fractions; 7–8, elution fractions containing recombinant TatD. Marker proteins (M) are shown at the left side.

Recombinant *E. histolytica* TatD shows Mg^{2+}-dependent endo-DNase activity

The DNase activity of recombinant TatD was tested with the closed circular form of pUC19 DNA (Figure 4A) or the linearised plasmid (Figure 4B) as substrate, and with the addition of different metal ions at various concentrations. TatD showed Mg^{2+}-dependent DNase activity, without $MgCl_2$ no substrate cleavage was observed. With 0.1 mM $MgCl_2$ some DNA degradation was visible on agarose gels, higher concentrations resulted in higher activities. Mn^{2+} also stimulated the activity but to a lesser extent than Mg^{2+} (data not shown). No DNase activity was observed with the addition of Ca^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and Fe^{3+} (data not shown). When the experiments were performed in various Mg^{2+}-containing assay buffers between pH 5 and pH 8, similar DNase activity was observed in all cases.

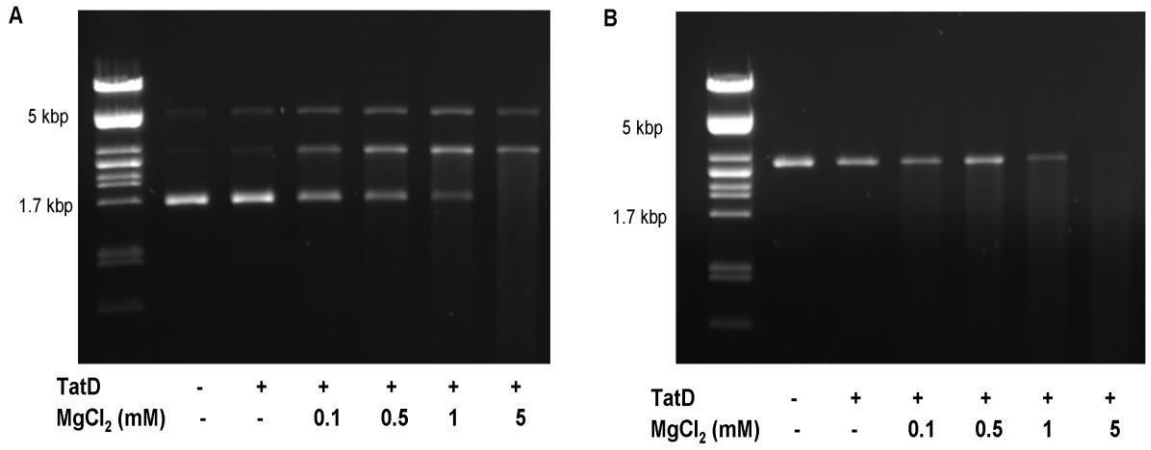

Figure 4. Nuclease activity of recombinant TatD using pUC19 plasmid as substrate. Panel A, incubation of the closed circular pUC19 plasmid with TatD (1 h, 37°C) at rising $MgCl_2$ concentrations showed increasing cleavage. With addition of 5 mM $MgCl_2$ a total degradation of the closed circular plasmid was observed and only linearised plasmid and nicked forms were visible. Panel B, incubation of the linearised plasmid with TatD plus $MgCl_2$ also showed DNA degradation, again the activity increased with higher $MgCl_2$ concentrations. Controls with plasmid incubated without TatD and plasmid incubated with TatD, but without metal salts, were included in each experiment.

DNase assay in native gels and immunoblot analysis

Nuclease activity of recombinant *E. histolytica* TatD was examined in a gel containing salmon sperm DNA as substrate. A single band was observed when 2.5 µg of recombinant enzyme or one unit of bovine DNase I were loaded (data not shown), however, this assay was not sensitive enough to show any signals for *E. histolytica* lysate (30 µg / lane) either from normal or from metronidazole-treated cells.

To visualise TatD, antisera against the recombinant protein were raised in rabbits and mice. A dilution series of recombinant TatD was loaded on an SDS-PAGE gel, blotted onto nitrocellulose, probed with the anti-TatD sera and bound antibodies were visualised with alkaline phosphatase-labeled second-

ary antibodies. Down to 1 ng of the recombinant TatD of 35 kDa could be detected (data not shown), but so far we were unable to detect the natural TatD in lysate from metronidazole-treated or control amoebae. These results suggest a very low level of TatD protein in the amoebae.

TatD localises to the cytoplasm of untreated and metronidazole-treated *E. histolytica* trophozoites

The cellular localisation of TatD was examined by confocal immunofluorescence microscopy using the mouse serum raised against the recombinant TatD. It was visualised with secondary antibodies labeled with Alexa 488 fluorescent dye, DAPI was used to stain the nucleus. Pre-immune serum was used as control (Figure 5, first row). In untreated amoebae, slightly granular cytoplasmic staining was observed (second row). After 2 h of 50 µM metronidazole treatment, most cells had rounded off, but the TatD labeling was largely unchanged (third row). After 4 h and 6 h, TatD was predominantly localised at the periphery along the plasma membrane (rows four and five). No relocalisation of TatD into the nucleus was observed in these metronidazole-treated cells. Rounding off and shrinkage of trophozoites became more pronounced between 4 – 6 h of metronidazole treatment, and after 12 h incubation period, most cells had detached from the slides. Nevertheless, few remaining cells could be examined (Figure 6). In some of these cells, TatD was not present in the nucleus (top row), whereas in others, we observed TatD fluorescence overlapping with the DAPI stain (bottom row).

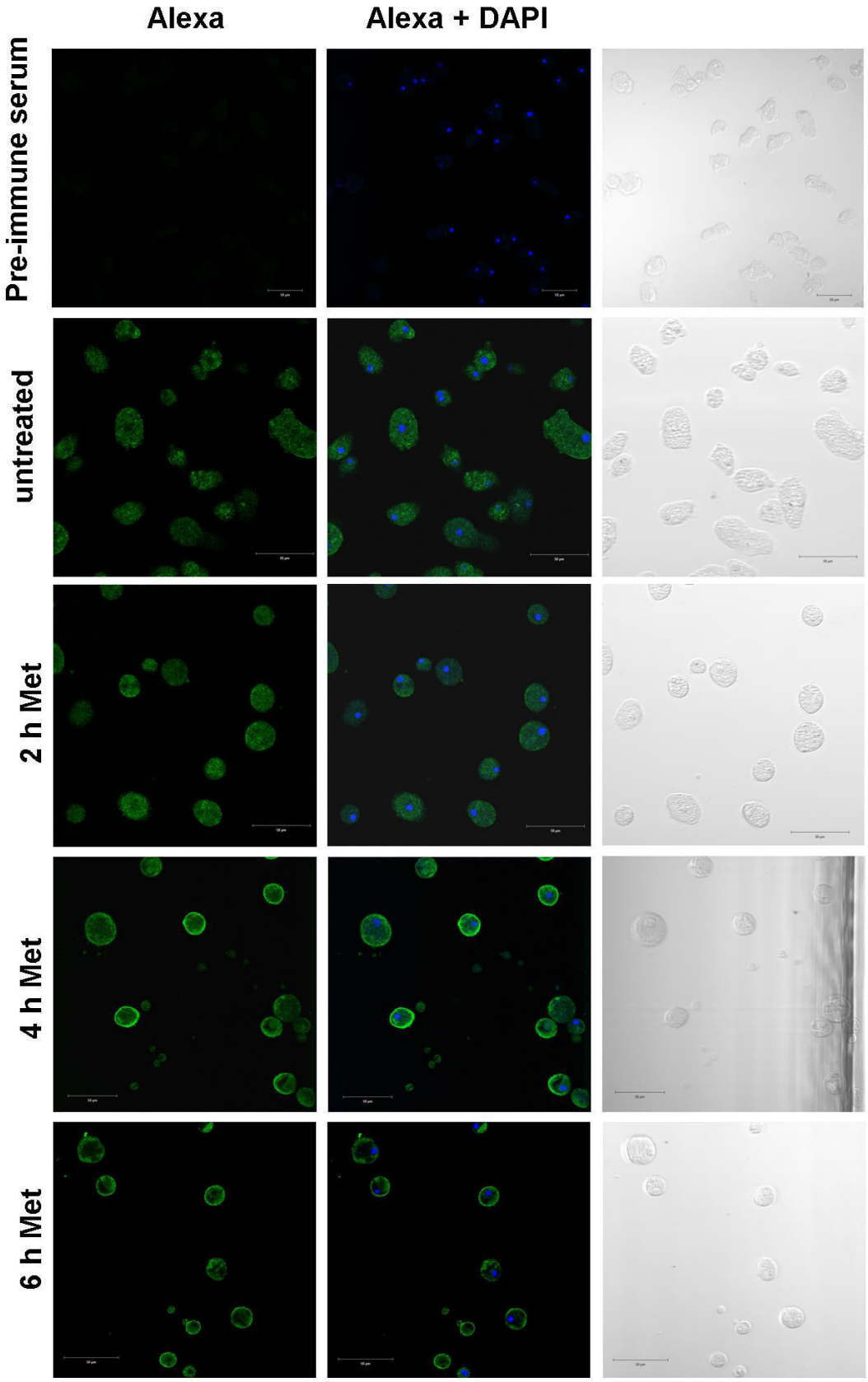
Alexa
Alexa + DAPI
Pre-immune serum
untreated
2 h Met
4 h Met
6 h Met

Figure 5. Cellular localisation of TatD in untreated and metronidazole-treated *E. histolytica* trophozoites by confocal immunofluorescence. Localisation was tested with anti-TatD mouse antiserum and Alexa 488-labeled secondary antibodies. The left and middle columns show TatD labeling (Alexa), with DAPI staining included in the middle column; the right column shows the corresponding phase contrast images. Cells incubated with pre-immune serum demonstrated no staining (first row) whereas in untreated amoebae granular cytoplasmic staining was observed (second row). Rows 3–5 show trophozoites treated for 2 h, 4 h, and 6 h with 50 µM metronidazole. After 4 h and 6 h, TatD labeling was localised more to the periphery along the plasma membrane. Bars in images represent 50 µm.

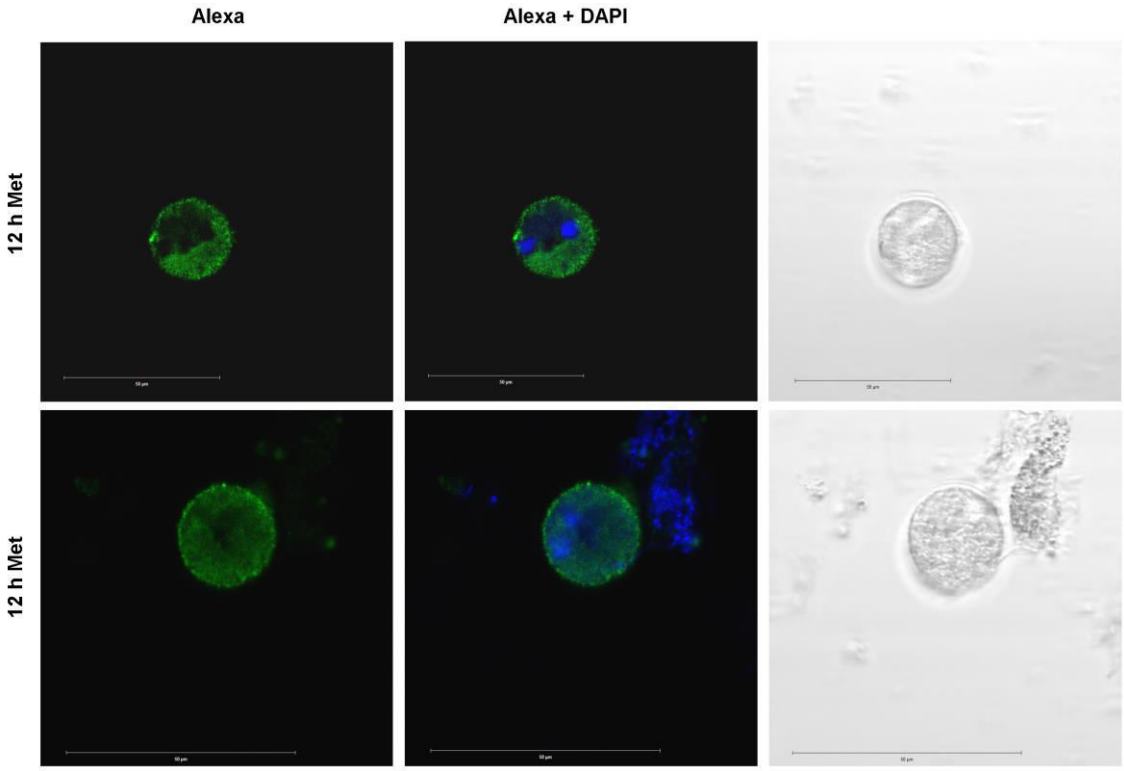

Figure 6. Cellular localisation of TatD in *E. histolytica* trophozoites treated with metronidazole for 12 h. Localisation was tested as above, but only very few cells remained to be evaluated. In some cells, TatD remained excluded from the nucleus (top row), whereas in others TatD labeling was found overlapping with the DAPI stain (bottom row). Bars in images represent 50 µm.

Towards a complete inventory of DNases in *E. histolytica*

We wanted to study the mRNA expression of TatD nuclease together with other putative endonucleases under the influence of metronidazole by qRT-PCR. With the availability of an annotated genome, it was possible to generate a list of proteins either annotated as nucleases or as carrying nuclease domains. We searched the NCBI (http://www.ncbi.nlm.nih.gov/protein/) and AmoebaDB (http://amoebadb.org/amoeba/) databases for DNases, RNases or nucleases, for this study, we focused on putative DNases. The list is given in Supplementary Table S1 and must be regarded as a work in progress, as it was not always trivial to distinguish between DNases and RNases. For example, five closely related candidates annotated as 5'-3' exonuclease domain containing protein were homologues of the essential Xrn1 RNases involved in cellular RNA turnover [44]. The situation can even be more complex, as Nakagawa and colleagues reported. Under certain conditions, DICER RNase can be converted to a death-promoting DNase [45], however, *E. histolytica* does not possess a full-length homologue of this factor. Other complex cases are the putative DNases encoded within LINEs (long interspersed elements). Mandal and colleagues [46] characterised one such DNase, which is the first discovered in *E. histolytica*, however, the protein sequence was not found in the *E. histolytica* databases. It had to be retrieved and assembled from genomic survey sequences, and was also added to Supplementary Table S1. The final version of this table now contains 38 DNases in total, 31 putative endonucleases and seven exonucleases. There are two large gene families, a divergent family of thirteen endonuclease / exonuclease / phosphatase genes and another family of seven closely related endonuclease V genes.

Up-regulation of few putative endonuclease genes but not the TatD gene is shown by qRT-PCR

For qRT-PCR experiments, we focused on the endonucleases and selected in total 16 genes for analysis: two different endonucleases III, one of the seven closely related endonucleases V, eight of the divergent endonuclease / exonuclease / phosphatase family members, a DNA repair endonuclease, a DNA excision repair protein, the LINE endonuclease, the GIY-YIG nuclease,

and TatD. Three independent experiments with metronidazole treated and untreated trophozoites were performed. Total RNA was extracted from the cultures and the mRNA expression of the putative endonucleases after 1 h and 4 h of treatment, as well as after 8 h and 12 h for the TatD mRNA, was determined. The efficiency of amplification was between 0.80 and 0.989. The final expression values and standard errors as analysed by the REST program are displayed in Supplementary Table 1. Eight of the 16 genes revealed significant changes (Figures 7 and 8). TatD expression did not change significantly after one hour of treatment, but then, unexpectedly, the expression dropped significantly to less than 50% and stayed low with a small increase after 12 h in comparison to the level at 8 h (Figure 7).

The mRNA expression of the endonucleases (Figure 8) displayed a complex pattern. The two endonuclease III mRNAs and the endonuclease V mRNA were down-regulated similar to the TatD mRNA after 4 h. Two members of the endonuclease / exonuclease / phosphatase family had more tendency towards up-regulation, one was significantly increased after 4 h, the other one as early as after 1 h. One DNA repair endonuclease was significantly down-regulated after 1 h and up-regulated after 4 h. Interestingly, the mRNA coding for the LINE endonuclease was up-regulated at both time points, especially after 4 h. The mRNA coding for GIY-YIG nuclease, another repair endonuclease, also appeared to be increased after both time points, but due to rather large variation of the values, this result was not included in Figure 8.

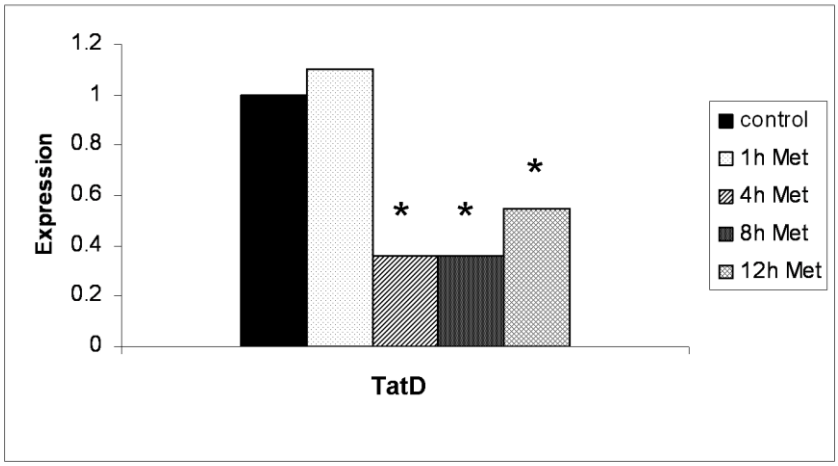

Figure 7. Expression levels of TatD after 50 µM metronidazole treatment. After one hour, TatD was slightly but not significantly up-regulated, whereas analysis showed a significant down-regulation after 4 h, 8 h and 12 h of metronidazole treatment.

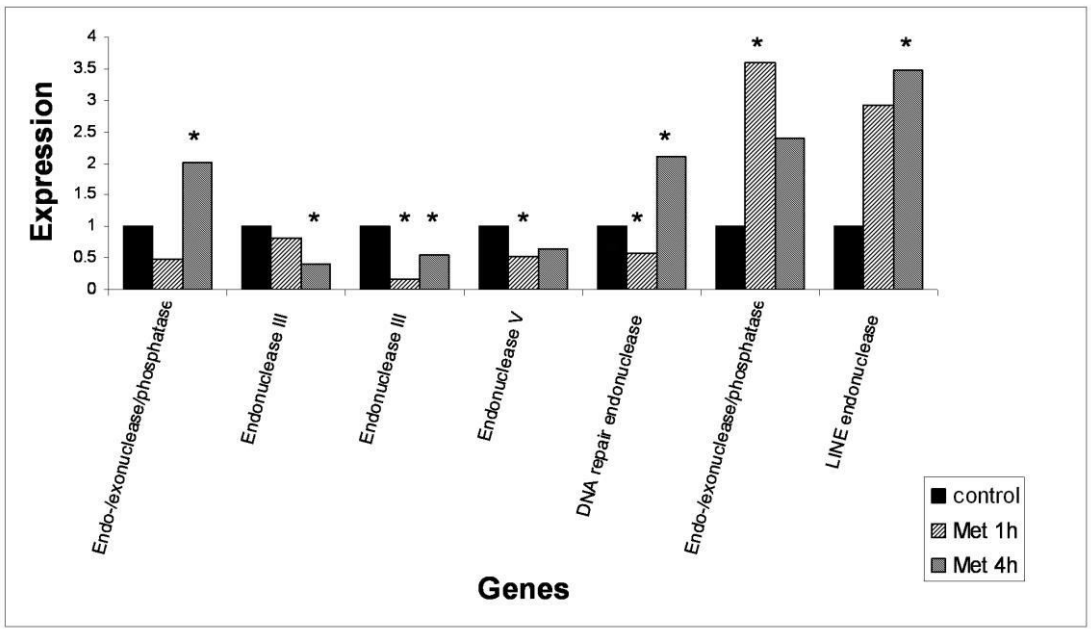

Figure 8. Changes in the expression of putative *E. histolytica* endonuclease genes after 50 µM metronidazole treatment. Whereas the two endonuclease III genes and the endonuclease V gene had a tendency towards down-regulation, the two genes belonging to the putative endonuclease / exonuclease / phosphatase family, a DNA repair endonuclease, and the LINE endonuclease showed a tendency towards up-regulation.

8.6 Discussion

When *E. histolytica* trophozoites are exposed to oxidative [21,22] or nitrosative [23] stress they die showing signs of degradation of chromosomal DNA. Similar observations were reported with G418 poisoning of amoebae [24] or with metronidazole treatment [K. Seifert, unpublished data, and this study]. This fragmentation of chromosomal DNA is typically observed in various species during apoptotic cell death. DNA degradation was also observed upon metronidazole exposure of *B. hominis* [26] or *G. duodenalis* [27,28], where an autophagy-like mechanism of programmed cell death was described [28].

The ultimate aim of our work is to understand how exactly DNA is degraded when *E. histolytica* is treated with metronidazole. As described above, our hypothesis was that enzymes in the parasite are responsible for this process. In higher eukaryotes, this apoptotic DNA degradation is elicited by various DNases [31], including caspase-activated DNase, the mitochondrial endonuclease G, the lysosomal DNase II and the ubiquitous cytosolic TatD nuclease. Our database search for these DNases showed that only TatD nuclease is present in *E. histolytica*, therefore we focused on this nuclease in the present study.

A recombinant expression plasmid based on pET-17b was constructed for the generation of TatD. Recombinant TatD was expressed in *E. coli* BL21-AI and purified under native conditions by metal chelate affinity chromatography (Figure 3). The recombinant TatD indeed had Mg^{2+}-dependent DNase activity (Figure 4). Mn^{2+} stimulated the activity to a lesser degree, but plasmid DNA was not degraded with addition of Ca^{2+}, Zn^{2+} or other ions. The enzyme was active over a rather broad pH range from pH 5 to pH 8. Extracts from *E. histolytica* trophozoites contained DNase activity (Figure 1), but so far we do not know how much TatD contributes to this activity. An activity gel containing DNA showed a band of the recombinant TatD protein, but with amoebic lysate, no bands were observed. So far, we were also unable to detect TatD in lysate by immunoblots using rabbit or mouse anti-TatD sera, while the recombinant protein could be detected down to 1 ng. These results suggest a very

low level of TatD protein present in *E. histolytica* trophozoites. It was, however, possible to detect TatD by confocal immunofluorescence microscopy (Figures 5 and 6, see below).

For all experiments on the effect of metronidazole on DNA degradation in *E. histolytica*, we chose a concentration of 50 µM, which is a lethal dose for the amoebae and less than the typical serum concentration in patients treated orally with the drug [47]. We analysed the fragmentation of chromosomal DNA by fluorescent TUNEL assay (Figure 2). TUNEL positive nuclei were observed after 12 h of incubation, and their number increased after further incubation, whereas nuclei in untreated cells did not become TUNEL-positive. This shows that DNA degradation is a rather late event under our conditions. The first process observed in 50 µM metronidazole treated cells is a rounding off within 90 min, but this process is not accompanied by cell death, as measured by Trypan blue exclusion. During the first two hours, metronidazole is reduced to reactive intermediates which covalently modify several thioredoxin reductase-associated proteins [7].

In our immunofluorescence study, the cytoplasmic TatD staining in the trophozoites appeared increased and was localised more towards the periphery after 4 h and 6 h (Figure 5). Up to 6 h, we did not observe any influx of TatD into the nuclei. Unfortunately, after longer incubation times, most of the rounded trophozoites detached and could no longer be stained. However, we were able to stain few remaining cells, treated for 12 h with metronidazole. Among these, we found cells where TatD remained excluded from the nucleus, and others where TatD was found to co-localise with the DAPI stain (Figure 6). This is tentative evidence for TatD in the nucleus after longer metronidazole treatment time. In addition, after 6 h of metronidazole treatment, the trophozoites displayed a reduced cytoplasmic volume expected for apoptosis-like cell death.

To test if the mRNA encoding TatD was up-regulated under the influence of metronidazole, we extracted RNA at several time points up to 12 h and performed qRT-PCR. Surprisingly, the level of TatD decreased significantly to about 40% of the initial level and stayed low even after 12 h (Figure 7). Superficially, it could make sense for the amoebae to down-regulate a potentially dangerous DNase in response to chemical stress. In partially metronidazole-

resistant *E. histolytica*, such a response to up-regulate protective enzymes and down-regulate enzymes contributing to the metronidazole damage was reported [4]. On the other hand, TatD down-regulation would appear to contradict the idea of an active process towards DNA degradation. These arguments based on the mRNA levels may not be sufficient, as post-translational effects, transport or membrane permeability or presence of inhibitors may also play a major role. Therefore more work is needed to understand how TatD nuclease acts in metronidazole-treated amoebae.

Although, in *E. histolytica*, TatD would be the prime candidate for an apoptotic DNase, the genome contains genes coding for many further DNases. However, these genes have not been described as being associated with apoptotic cell death. Overall, we identified 31 endonucleases including TatD. These included two large gene families, one family of thirteen variable endonuclease / exonuclease / phosphatase genes and one of seven closely related endonuclease V genes. In addition, seven exonuclease genes were identified.

We selected 16 endonuclease genes for qRT-PCR analysis of untreated trophozoites vs. 1 h or 4 h metronidazole-treated cells, eight of which including TatD showed significant expression changes (Figures 7 and 8). Besides TatD, two endonuclease III and one endonuclease V family member were down-regulated. The two endonucleases are annotated as belonging to the DNA glycosidases, and endonuclease V is annotated as an enzyme initiating repair of nitrosatively deaminated purine bases. The other four endonucleases were all up-regulated after 4 h. The up-regulated gene product annotated as DNA repair endonuclease is a homologue of yeast RAD1 and human XPF, which are the DNases in the large nucleotide excision repair complexes [48]. Various DNA repair systems also exist in *E. histolytica*, but they are missing some of the components present in higher eukaryotes [49]. The GIY-YIG domain nuclease was found up-regulated, though not significantly. The protein is similar to human SLX1, which is part of the SLX1-SLX4 MUS81-EME1 complex which resolves branched DNA intermediates [50]. In *E. histolytica* only SLX1 is found, whereas yeast possesses homologues of all four components. At this time the function of this single factor in *E. histolytica* is unknown.

The two members of the “endonuclease / exonuclease / phosphatase family“ showed more tendency towards up-regulation, one was significantly in-

creased after 4 h, the other one as early as after 1 h. By now, it can be questioned, however, whether this family of proteins contains any DNases. Whereas this annotation is still present in the AmoebaDB database, the two genes are now annotated as neutral sphingomyelinase (XP_652152) and phospholipase C precursor (XP_654810) in the NCBI database, and may not have any nuclease activity at all.

Finally, an interesting case to be discussed is the LINE endonuclease, the first DNase characterised in *E. histolytica* [46] and, in comparison, in *E. dispar* [51]. Unfortunately, no annotated sequence exists, neither in the NCBI nor in the AmoebaDB databases, but with help of the original publication, the gene could be retrieved from genomic survey sequences at the NCBI database (Supplementary Table S1). Interestingly, the mRNA coding for the LINE endonuclease was up-regulated in metronidazole-treated trophozoites, both at 1 h and 4 h. So far it is not known, if the LINE nuclease contributes to the baseline DNase activity in *E. histolytica* and whether this nuclease may play any role in the DNA degradation upon metronidazole treatment.

Our study suggests TatD as a candidate for DNA degradation during metronidazole toxicity. Although TatD remained in the cytoplasm until 6 h of metronidazole treatment, we were able to obtain tentative evidence that in 12 h metronidazole-treated trophozoites TatD could be found in the nucleus, however, only within few remaining cells, after many damaged cells had detached.

Although our data point to TatD, this is not necessarily the only DNA damaging agent. For example, the LINE endonuclease should also be considered. Moreover, it will be necessary to understand the ability of *E. histolytica* to repair damaged DNA, and whether the cells perform a controlled switch from trying to cope with DNA degradation to apoptosis-like cell death.

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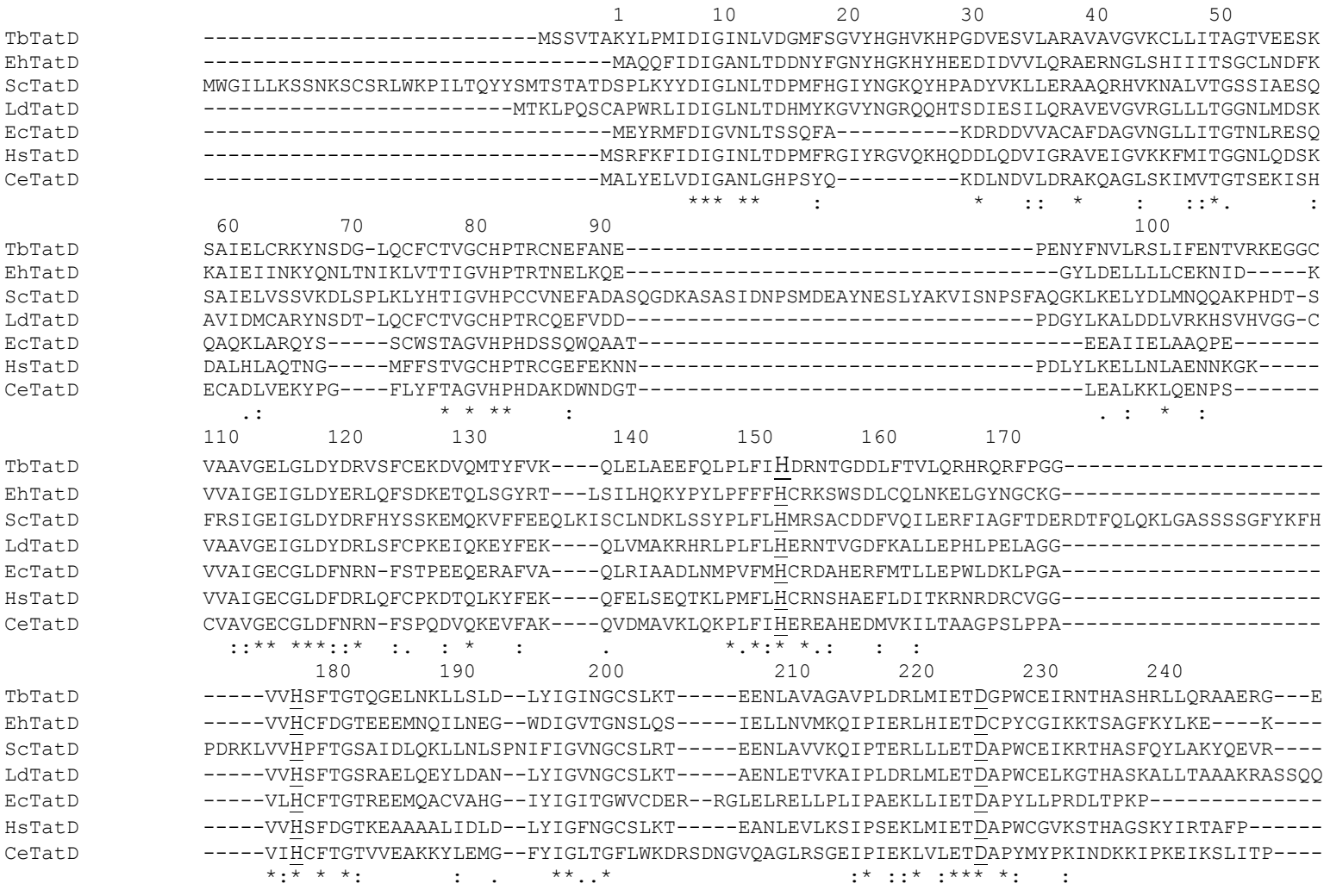
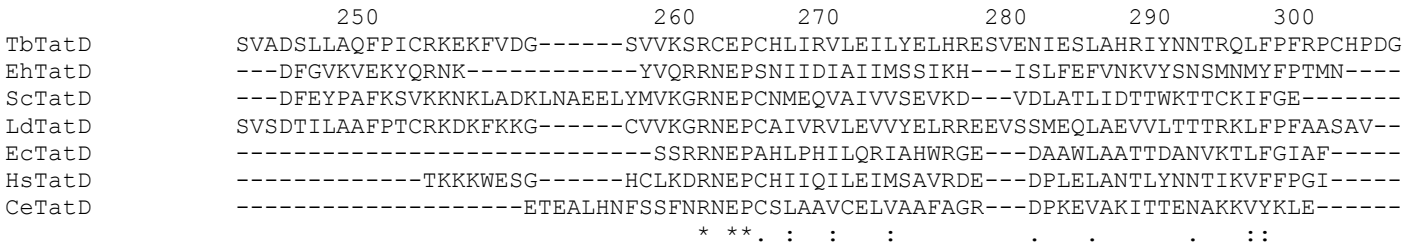
Supplementary





Supplementary Figure S1: Comparison of TatD proteins from various species. The residues were numbered on the basis of the *E. histolytica* sequence. EhTatD (XP_651470), *E. histolytica*; TbTatD (XP_828684), *Trypanosoma brucei* [38]; ScTatD (AAT92645), *Saccharomyces cerevisiae* [35]; LdTatD (XP_003859100) *Leishmania donovani* [36]; EcTatD (CAA06727) *E. coli* [33]; HsTatD (AAH64964) human; CeTatD (NP_504476), *Caenorhabditis elegans* [34]. The residues important for enzyme actvity His151, His176 and Asp224 [38] are underlined.

Supplementary Table S1: Putative DNases encoded in the *E. histolytica* genome. The protein accession numbers from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/) are shown, then the predicted molecular masses and the predicted isoelectric points of the proteins. The last column contains the oligonucleotide probes for the qRT-PCR of selected genes. Mtz Expr means the relative expression values for 1 h or 4 h treated trophozoites as calculated by the REST program. Std+ and Std- are the respective standard errors.

No	Name	Accession	Predic-ted MW [kDa]	Predic-ted pI	1 h Mtz Expr	1 h Std -	1 h Std +	4 h Mtz Expr	4 h Std -	4 h Std +	Comments	Oligonucleotides: top, forward; bottom, reverse
	Endonucleases											
1	Endonuclease III	XP_654116	35.6	6.04	0.168	0.084	0.348	0.551	0.329	0.919		ATGTCAAAAGCATCCGAAGA CGTTGGTCAACAGTAATACCTTC
2	Endonuclease III	XP_655025	27.5	8.85	0.816	0.444	1.551	0.419	0.243	0.74		CAACGACTAGGTTGGGCAGA TCCAAAAGCAACAAGCGACT
3	Endonuclease V	XP_647849	26.6	6.44	0.519	0.355	0.887	0.648	0.444	1.116		TGCTTGTTGATGGAAATGGA TCACCTGGTTTGATTTGTGAA
4	Endonuclease V	XP_648272	26.5	6.24							Endonu-clease similar to 1	
5	Endonuclease V	XP_649401	26.5	6.44							Endonu-clease similar to 1	
6	Endonuclease V	XP_651871	26.6	6.44							Endonu-clease similar to 1	
7	Endonuclease V	XP_653952	26.6	6.07							Endonu-clease similar to 1	
8	Endonuclease V	XP_001913899	26.6	6.45							Endonu-clease similar to 1	
9	Endonuclease V	XP_001914306	26.6	6.44							Endonu-clease similar to 1	
10	TatD	XP_651470	35.0	6.08	1.105	0.381	2.501	0.96	0.366	2.402	Putative apoptotic DNase	TGGTGTTCATCCAACTCGTACA TCCAATTTCTCC-TATAGCCACAAC
11	FEN-1 nuclease	XP_651270	42.6	8.9							Flap En-donucle-ase-1	

12	Endonuclease/Exonuclease/phosphatase family	XP_649653	92.7	8.62	1.394	0.445	4.63	1.484	0.562	3.365	Re: inositol polyphosphate 5-phosphatase	CAAGCATCAGTTCGCACTACC TGGAGATGGTGGGGTTGTAG
13	Endonuclease/Exonuclease/phosphatase family	XP_652152	43.8	5.02	0.863	0.673	1.14	0.847	0.573	1.223	Re: neutral sphingomyelinase	TGGGAAAGTGGTGAAACTGAA TTGGATGACGATGAAGACCA
14	Endonuclease/Exonuclease/phosphatase family	XP_654810	35.2	6.40	3.583	1.347	11.21	2.396	0.851	6.976	Re: phospholipase C precursor	TCCCATTGTAAGTCAACACCA TGGATCATTGTCATGTGCTG
15	Endonuclease/Exonuclease/phosphatase family	XP_001913433	35.2	6.36							Re: phospholipase C precursor	
16	Endonuclease/Exonuclease/phosphatase family	XP_001913468	34.2	6.5							Re: phospholipase C precursor	
17	Endonuclease/Exonuclease/phosphatase family	XP_001914105	35.2	6.4							Re: phospholipase C precursor	
18	Endonuclease/Exonuclease/phosphatase family	XP_649417	35.2	6.40	1.797	0.63	5.823	1.655	0.609	3.89	Re: inositol polyphosphate 5-phosphatase	AAACAACTAACCCCTCGAACAA ACGTCTTGGGATTGGTTTTG
19	Endonuclease/Exonuclease/phosphatase family	XP_649962	79.4	6.09	1.862	0.352	11.62	1.799	0.356	5.111	Re: inositol polyphosphate 5-	TGTTCAAAATGATGGAAATGC GGTTCAATATGTAACCAATCTG-GA

											phospha-tase	
20	Endonuclease/Exonuclease/phosphatase family	XP_650540	107.9	8.07	0.469	0.179	1.649	2.002	0.974	4.419	Re: inositol polyphosphate-5-phosphatase	GCACCAATGAGTTGGATTCTT CCTCGACTAAAAACGCATTGA
21	Endonuclease/Exonuclease/phosphatase family	XP_651096	77.4	5.48	1.051	0.247	3.167	1.677	0.551	4.513	Re: inositol polyphosphate 5-phosphatase	TGGGGTTATGACATTTGGACA TGCTCCTTTATTTGCCATTCC
22	Endonuclease/Exonuclease/phosphatase family	XP_656367	88.6	5,28	0.557	0.22	1.243	0.718	0.415	1.226	Re: inositol polyphosphate 5-phosphatase	CGTCCAGACAGTAAATTGGTTTG TGCTTGTTCTGGTTGGATGA New oligonucleotides
23	Endonuclease/Exonuclease/phosphatase domain containing protein	XP_656286	37.2	7.92							Re: neutral sphingomyelinase	
24	Endonuclease/Exonuclease/phosphatase domain containing protein	XP_656499	37.0	6.14							Re: Neutral sphingomyelinase	
25	DNA repair endonuclease	XP_657509	104.7	6.51	0.568	0.287	1.042	2.106	1.516	3.449	Homologous to yeast RAD1, human XPF	TGACTCGGAACCAACTGAAGA AATTCCAGGCATTCGTTTCA

26	DNA excision repair protein	XP_652464	27.9	9.46	0.596	0.347	1.043	0.778	0.522	1.108		GGGATAAACTCGCAAAATGC TTGGGGAATGAAAGACTCTCC
27	LINE endonuclease	AZ541065 random sequence and ENTDB02TR genomic survey sequence	28.4	8.76	2.913	1.088	11.72	3.476	1.271	7.971	protein not annotated	AAAACCCCCATATGAAACAGTG CTTTTCTTCCAATAGGCTCCA
28	GIY-YIG Nuclease	XP_652913	35.9	9.23	2.971	0.617	30.28	2.568	0.679	28.45	Homologous to human SLX1	GTGGGGGTGCTTTCAAAACT ATGTTGCCAATCCCATTCAA
29	A/G-specific adenine glycosylase	XP_653060	35.5	9.45								
30	DDE superfamily endonuclease	XP_653824	58.1	9.15								
31	Serine/threonine protein kinase 6	XP_655957	59.1	8,49							Carries an endonuclease V domain	
	Exonucleases											
32	Exonuclease I	XP_648209	56.5	5.82							Exonuclease I	
33	Exonuclease I	XP_655654	55.5	5.14							Exonuclease I	
34	Exodeoxyribonuclease III	XP_650532	37.4	8.64							Exonuclease III	
35	RecQ DNA helicase exhibiting an exonuclease activity	XP_657471	32.0	6,49							DEDDy 3'-5' exonuclease domain	

36	DNA polymerase alpha catalytic subunit	XP_657373	130.2	8,7							DEDDy 3'-5' exonu-clease domain	
37	DNA polymerase delta catalytic subunit	XP_654477	124.4	8.3							DEDDy 3'-5' exonu-clease domain	
38	DNA polymerase zeta catalytic subunit	XP_656768	161.9	5,65							DnaQ-like (or DEDD) 3'-5' exo-nuclease domain	

9. Additional biochemical analysis of *E. histolytica* TatD

9.1 Introduction

The manuscript submitted to PloS Neglected Tropical Diseases, entitled "*Entamoeba histolytica*: Molecular Characterisation of a DNase Homologous to Bacterial TatD", received reviews requiring further experiments. The main point raised by two of the reviewers was that the recombinant TatD was not purified enough to exclude that the DNase activity could be present in contaminating proteins. Therefore, further purification steps were requested. To respond to this query, two different approaches were taken. First of all, mutant TatD proteins were produced, with the argument that if important residues are changed, and the DNase activity of recombinant TatD disappears, then the activity is associated with TatD and not with the contaminants. In a second approach, a further purification step of the recombinant *E. histolytica* TatD was performed by ion exchange chromatography, and DNase activity was measured again. To aid this, a fluorescence DNase activity assay using a beacon probe was carried out.

Taken together, a requested control experiment for the immunofluorescence could be provided, but the results of the additional experiments performed on mutant and purified recombinant TatD raised significant doubts whether TatD really is a DNase.

9.2 Materials and methods

Site-directed mutagenesis of *E. histolytica* TatD

The QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) was used to introduce the following mutations into the recombinant TatD: H81A, E113A, H151A, H176A, E222A and D224A according to the manufacturer's instructions. The *E. histolytica* TatD residues H151, H176 and D224 are reported to be important for enzyme activity (Gannavaram and Debrabant 2012), and are conserved in *T. brucei*, *L. donovani*, *S. cerevisiae*, *C. elegans*,

E. coli as well as in human TatD. Moreover, proteins containing the mutant residues H81A (active site), E113A and E222A (putative Mg^{2+}-binding sites) were produced.

The PCR run protocol was adjusted as follows: 95°C for 30 s, 16 cycles of (95°C, 30 s; 56°C, 60 s and 68°C, 5 min). Table 2 shows the forward and reverse primers used for PCR to generate the TatD mutants. Mutations were confirmed by sequencing with T7 and T7term primers (Microsynth).

Cloning, expression and purification of recombinant TatD wild type (WT) and mutant gene products were performed as described in the manuscript.

Table 2 Forward (F) and reverse (R) primer sequences to generate the TatD mutant proteins H81A, E113A, H151A, H176A, E222A, D224A.

Forward (F) and reverse (R) primer sequences to generate TatD mutant proteins	
H81A (F)	5'-GTT ACT ACA ATC GGT GTT GCG CCA ACT CGT ACA AAT GAG-3'
H81A (R)	5'-CTC ATT TGT ACG AGT TGG CGC AAC ACC GAT TGT AGT AAC-3'
E113A (F)	5'-GTT GTG GCT ATA GGA GCG ATT GGA TTA GAT TAT G-3'
E113A (R)	5'-CAT AAT CTA ATC CAA TCG CTC CTA TAG CCA CAA C-3'
H151A (F)	5'-GCC TTT CTT CTT TGC GTG TAG AAA ATC TTG G-3'
H151A (R)	5'-CCA AGA TTT TCT ACA CGC AAA GAA GAA AGG C-3'
H176A (F)	5'-CTG TAA AGG AGT TGT TGC GTG TTT TGA TGG AAC AG-3'
H176A (R)	5'-CTG TTC CAT CAA AAC ACG CAA CAA CTC CTT TAC AG-3'
E222A (F)	5'-GAA CGT CTT CAT ATT GCG ACT GAT TGT CCT TAT TG-3'
E222A (R)	5'-CAA TAA GGA CAA TCA GTC GCA ATA TGA AGA CGT TC-3'
D224A (F)	5'-CTT CAT ATT GAA ACT GCG TGT CCT TAT TGT GG-3'
D224A (R)	5'-CCA CAA TAA GGA CAC GCA GTT TCA ATA TGA AG-3'

Gel-based assay for nuclease activity

For the comparison of the nuclease activities of WT and mutant TatD proteins, the proteins were purified under native conditions with Ni-NTA spin columns as described in the manuscript. pUC19 plasmid DNA (1 µg) was used as substrate and incubated with 1 µg of recombinant *E. histolytica* TatD (WT and mutants H81A, E113A, H151A, H176A, E222A, D224A) for 1 h at 37°C in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.0. Samples were analyzed by electrophoresis on 1% agarose gels with ethidium bromide staining followed by UV illumination.

Ion exchange chromatography

Recombinant WT TatD and the mutant E113A protein were purified under native conditions with Ni-NTA spin columns as described above. Then, a second purification step was performed using cation exchange chromatography. This purification step was done at the laboratory of Rudolf Glockshuber (Institute of Molecular Biology and Biophysics, ETH Zürich, Switzerland), carried out by Christoph Giese. The recombinant proteins were loaded onto a 1 ml Resource S column (GE Healthcare Life Sciences) at 4°C in 20 mM MES-NaOH, pH 6.0, and eluted with a 0-400 mM NaCl gradient over 30 ml. The peaks were validated by mass spectrometry.

Further experiments were then conducted in Vienna with these purified fractions.

TatD activity measurements after ion exchange chromatography

First, a gel-based assay was carried out as described above. Second, TatD activity was analyzed with a self-designed molecular beacon in a fluorescence based DNase detection assay (Biggins et al. 2000). The beacon had a stem-loop structure with a fluorophore (FAM) and a quencher (BHQ-1) linked to its 5′- and 3′-ends, respectively (5′- FAM GCT ATT AGA ATT CTT GAA TTT TTT TTT TTT CAA GAA TTC TAA TAG C BHQ-1 -3′, Fig. 5). When DNases cut the stem, the formerly quenched fluorescence of FAM is restored which results in an increase of fluorescence.

The beacon probe (final concentration 5 µM) was incubated with 1 µg of purified recombinant *E. histolytica* WT TatD or E113A mutant for 1 h at 37°C in DNase I buffer (Thermo Scientific), in a 20 µl volume. For fluorescence measurements, buffer was added to a final volume of 100 µl. One unit of bovine DNase I (Thermo Scientific) was used as positive control. Fluorescence measurements were performed with a fluorescence microplate reader (Tecan GENios), with an excitation wavelength of 490 nm and an emission wavelength of 550 nm. For the analysis, the Magellan™ - data analysis software was used. Experiments were repeated twice and mean values were calculated. Background fluorescence, observed without enzyme addition, was subtracted.

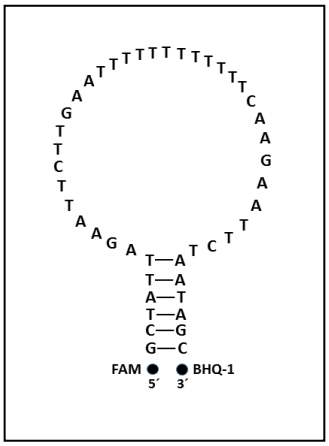


Fig. 5 Stem-loop structure of the molecular beacon. The fluorophore FAM is linked to the 5′-end whereas the quencher BHQ-1 is linked to the 3′-end. DNases cut the stem and the fluorescence is restored.

9.3 Results

Nuclease activity of the TatD mutants

Surprisingly, recombinant TatD WT and the mutant proteins H81A, E113A, H151A, H176A, E222A, D224A all showed nuclease activities with plasmid pUC19 used as substrate; however, some mutants were more active than others (Fig. 6). TatD H81A showed the highest activity of all the tested mutants, with no supercoiled pUC19 visible on the gel any more. Mainly open circular plasmid DNA was observed. TatD E113A had the weakest activity with only small amounts of open circular pUC19. The other mutant proteins revealed similar activities as the wild type TatD. Supercoiled pUC19 plasmid DNA without any addition of TatD was used as control.

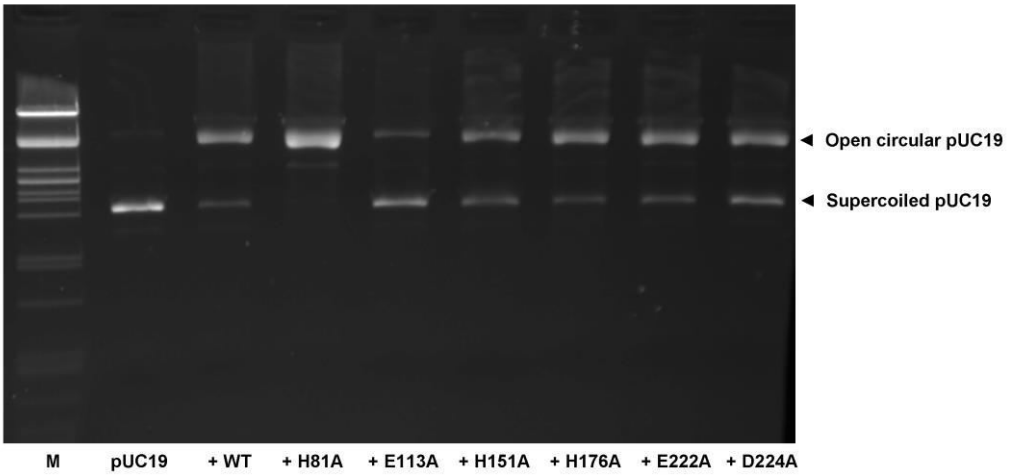


Fig. 6 Nuclease activities of recombinant TatD WT and the mutant proteins using pUC19 plasmid as substrate. The TatD WT protein had similar activity as the mutants H151A, H176A, E222A and D224A. H81A revealed the highest activity, whereas E113A showed the weakest activity. The Lambda DNA/PstI marker (M) is shown on the left side.

As all the recombinant TatD variants displayed nuclease activity, it had to be suspected, that *E. coli* nucleases were co-purified with the metal chelate chromatography. Therefore, it was examined if a preparation of recombinant *E. histolytica* fructokinase, purified by Ni-NTA affinity chromatography as well, also had such an activity. Indeed, the recombinant fructokinase also showed nuclease activity (data not shown), similar to WT TatD. This clearly showed that all recombinant proteins including the recombinant WT and mutant TatD proteins contained the *E. coli* nuclease contamination. Therefore, it was necessary to carry out further purification. In a cooperation with Christoph Giese and Rudolf Glockshuber from the ETH Zurich, a second purification step by means of cation exchange chromatography was performed on the recombinant WT and E113A mutant TatD proteins.

Nuclease activity of ion exchange chromatography-purified WT TatD and TatD E113A

After ion exchange chromatography, nuclease activities of TatD WT and E113A were examined again using the pUC19 cleavage assay. Compared to the control with only pUC19 plasmid DNA incubated, the activity was very weak (Fig. 7); in fact, the products were almost not visible on the ethidium-bromide stained agarose gel. Moreover, both WT TatD and the mutant E113A showed the same weak activity.

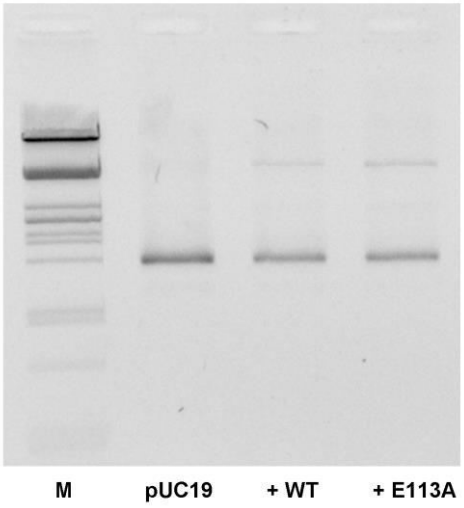


Fig. 7 Nuclease activity of WT TatD and E113A after ion exchange chromatography. Only a very weak activity of both purified proteins was observed with pUC19 DNA as substrate.

Fluorescence activity measurements

In order to be able to better quantify the DNase activity, a fluorescence assay was established. A self-designed molecular beacon was used containing a FAM molecule directly adjacent to a BHQ-1 quencher (Fig. 5) (Biggins et al. 2000).

The fluorescence assay showed almost no DNase activity of the purified WT TatD or the mutant E113A (Fig. 8). In contrast, bovine DNase I showed a very high fluorescence and therefore high nuclease activity.

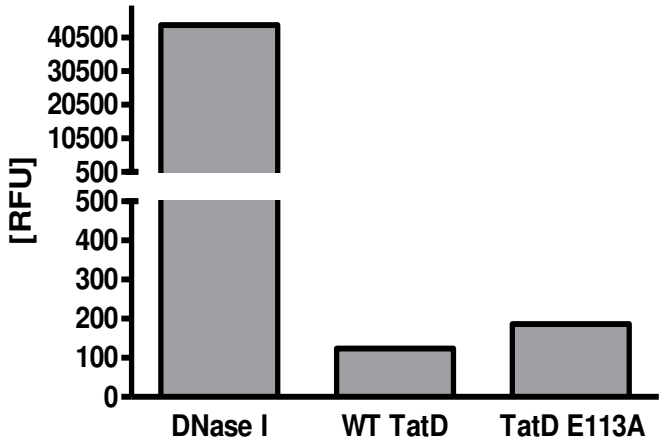


Fig. 8 Fluorescence measurements of nuclease activity using a molecular beacon. Bovine DNase I showed a very high DNase activity, in contrast to WT TatD and E113A, which had almost no activity. RFU are relative fluorescence units.

Structure and sequence analysis of *E. histolytica* TatD

For a better understanding of the apparent lack of TatD activity, the crystal structure of *E. histolytica* TatD was useful (PDB ID: 3IPW; Edwards et al., unpublished). With the help of Guido Capitani (Laboratory of Biomolecular Research, Paul Scherrer Institute, Villigen, Switzerland), the *E. histolytica* and *E. coli* TatD structures (PDB ID: 4P5U; Chen et al. 2014) were visually compared to find differences that might be responsible for the activity loss in *E. histolytica*. Instances of differing residues include (S10>D14), (H64>T83), (D65>R84), (W176>N200) and (L207>C228), with the *E. coli* variant written first.

A further validated nuclease activity of the TatD protein with a known protein structure was found in *S. cerevisiae* (Qiu et al. 2005; structure PDB ID: 3E2V; Bonanno et al., unpublished). The *E. histolytica*, *E. coli*, yeast and many other homologous TatD proteins from both eukaryotic and prokaryotic organisms were aligned using the Expresso algorithm in T-Coffee, a multiple sequence alignment tool (Notredame et al. 2000). The *E. histolytica* TatD residues D14 and C228 are also found in yeast, so it is unlikely that they were causing the activity loss. T83 and R84 show some interesting differences: they are found as C116 and C117 in *S. cerevisiae* and as H64 and D65 in *E. coli*. However, the *Entamoeba* proteins match all non-yeast eukaryotic homologs. The fact

that the protein has remained like this across so many eukaryotic species suggests that these residues are not causing the lack of activity in the *E. histolytica* homolog.
In contrast, the N200 residue appears as an asparagine in all *Entamoeba* TatD proteins, but is found as a cysteine in all other eukaryotes, and as a tryptophan in all prokaryotes. Considering the proximity of the residue to the active site, this is likely to have an influence on activity. Therefore, the N200 residue could be mutated to cysteine or tryptophan for future investigations on the TatD activity.

With regard to proteins of similar structure and different function, a DALI (distance alignment matrix method) search revealed two interesting PDB entries. The amidohydrolase from *Proteus mirabilis* and the resiniferatoxin-binding protein from *Rhodobacter capsulatus* are similar in structure to TatD but have other functions. Therefore, it is indeed conceivable that the *E. histolytica* TatD could have evolved to a function other than a DNase, maybe to a function as a binding protein.

9.4 Discussion

Analysis of the hypothetical *E. histolytica* TatD DNase activity

The central reviewer query for the TatD manuscript was whether the recombinant TatD protein was purified enough to be sure that it was responsible for the observed DNase activity. Two approaches were chosen to address this query.
In the first approach, different point mutations, including conserved residues and residues in the active site, were introduced into TatD. At least some of these mutant proteins were expected to lose the nuclease activity of the *E. histolytica* TatD. Surprisingly, all the mutant proteins showed more or less activity (Fig. 6). This was the first hint that the measured DNase activity could reside in a contamination of the recombinant TatD. Even more disturbingly, recombinant *E. histolytica* fructokinase, also purified by metal chelate chroma-

tography, had a similar DNase activity as the TatD WT and some mutants, which should not have been found in this metabolic enzyme.
These experiments also showed a more general problem. If *E. coli* DNases bind Mg^{2+} required for their activity, it has to be expected that these DNases weakly bind to metal chelate columns too, and will be eluted with any recombinant protein isolated this way. In the future, the use of these columns could be circumvented, for example by producing the recombinant TatD with a recently developed metal-independent Twin-Strep-tag protocol (Schmidt et al. 2013).

In the second approach, a further purification step of the recombinant *E. histolytica* TatD wild type and the TatD E113A mutant, which had shown the weakest activity, was performed by cation exchange chromatography. After the second purification step, the activity should have been enriched but the purified preparations only had a very weak DNase activity in the nuclease cleavage assay using pUC19 as substrate. This weak activity was about the same in TatD WT and E113A (Fig. 7). As it was unlikely that an activity loss was caused by the execution of ion exchange chromatography, our nuclease activity was probably observed due to a contamination of the recombinant TatD protein fraction after the first purification by metal chelate affinity chromatography (Fig. 3, TatD manuscript). This activity could have been caused by *E. coli* TatD, but other contaminants cannot be excluded. The observed very weak activity after the ion exchange step is a background activity, maybe still caused by a contamination. On the other hand, the possibility of a low nuclease activity of *E. histolytica* TatD cannot be ruled out completely.
In addition to the plasmid cleavage assay, fluorescence measurements with a FAM-linked beacon were performed with the enriched TatD WT and E113A mutant (Fig. 8). In this quantitative assay, only a weak TatD nuclease activity was observed, in contrast to bovine DNase I which showed a strong fluorescence (356-fold the activity of WT TatD and 238-fold of the E113A mutant). This result strengthened the suspicions raised through the previous experiments: TatD is probably no DNase.
In *S. cerevisiae*, not only an endonuclease but also an exonuclease activity of TatD was described by Qiu et al. (2005), and Chen et al. (2014) suggested a

3'-5' exonuclease activity of *E. coli* TatD. It is not quite clear, if the fluorophore FAM could interfere with a possible exonuclease activity in our beacon assay. However, analyses of the ability of different exonucleases to cleave a FAM-linked DNA substrate have shown that at least those exonucleases tested were able to do so (New England Biolabs 2015: https://www.neb.com/tools-and-resources/selection-charts/properties-of-exonucleases-and-endonucleases). In this work, the possibility of an exonuclease activity was not pursued any further.

Analysis of the hypothetical role of *E. histolytica* TatD in DNA destruction after metronidazole treatment

Confocal immunofluorescence experiments revealed a cytoplasmic distribution of TatD, but it could not definitely be found localized to the nucleus after metronidazole treatment (Fig. 5 and Fig. 6, TatD manuscript). This result would confirm the doubts about an apoptotic nuclease function of the amoebic TatD. In western blot analysis, TatD was not detected in *E. histolytica* lysates. Moreover, in TUNEL experiments with recombinant TatD directly applied to the slides, no positive *E. histolytica* cells were observed, meaning no chromosomal DNA fragmentation. In contrast, TUNEL-positive cells were observed when recombinant *T. brucei* TatD was applied onto *T. brucei* cells (Gannavaram and Debrabant 2012). Both experiments were carried out with TatD only purified by metal chelate affinity chromatography, so these results have to be questioned too.

When the mRNA expression of TatD after metronidazole treatment of *E. histolytica* was examined by qRT-PCR, no up-regulation was observed, in contrast, the mRNA encoding TatD was significantly down-regulated after 4 h, 8 h and 12 h, respectively (Fig. 7, TatD manuscript). This result stands in contrast to what is expected for an apoptotic DNase after the induction of chemical stress and adds more doubts to the idea of metronidazole-induced DNA damage by TatD. On the other hand, it could be conceivable that *E. histolytica* down-regulates a potentially damaging factor. The down-regulation of thioredoxin reductase, a metronidazole-activating enzyme, under metronidazole pressure would be such an example (Wassmann et al. 1999).

The role of TatD in other organisms

TatD was initially identified as the product of an open reading frame in the *E. coli* twin arginine transport (*tat*) operon (Sargent et al. 1998), but soon after, Wexler et al. (2000) described the function of an endo-DNase. Recently, an additional 3'-5' exonuclease activity was found which preferentially digests single-stranded DNA and RNA. Further, the authors suggested a function in DNA repair (Chen et al. 2014). Whereas Wexler et al. (2000) purified *E. coli* TatD from an overexpressing strain by two consecutive anion exchange chromatography steps followed by gel filtration, and did not use a hexahistidine tag, Chen et al. (2014) used this technology and purified the product by one further ion exchange chromatography step, similarly as performed in this work.

Overall, TatD represents an evolutionary conserved protein found in prokaryotic and eukaryotic organisms. We focused on TatD out of the reason that it was the only endonuclease found in *E. histolytica* which was characterized as apoptotic nuclease in several other species. Homologs of this protein with the function of an apoptotic nuclease were described in *C. elegans* (Parrish and Xue 2003), *S. cerevisiae* (Qiu et al. 2005), *Leishmania* (BoseDasgupta et al. 2008), and *T. brucei* (Gannavaram and Debrabant 2012).

In *C. elegans*, a complex biochemical process was described, which involves multiple nucleases and non-nuclease cofactors (Parrish and Xue 2003). CPS-6, an endonuclease G homolog (Parrish et al. 2001), and NUC-1, which encodes a type II DNase, were found to play a role in apoptotic DNA degradation. In addition, seven other cell-death-related nucleases (CRNs) were found (Parrish and Xue 2003). The authors suggested the existence of at least two independent pathways in *C. elegans,* which participate in apoptosis and DNA degradation. CRN-1 is homologous to the human flap structure-specific endonuclease 1 (FEN-1), which normally cuts short single-stranded DNA overhangs. The *E. histolytica* FEN-1 homolog could also play a role in DNA degradation in amoebae. However, as our focus was directed towards apoptotic endonucleases in the beginning, FEN-1 was not part of our investigation. Nevertheless, it would be interesting to take a closer look at this protein and its function in *E. histolytica*.

In yeast, eight apoptotic nucleases analogous to those described in *C. elegans* were found, including the TatD protein (Qiu et al. 2005). TatD depleted *T. brucei* treated with prostaglandin D2 (PGD2) or H_2O_2 showed decreased amounts of TUNEL-positive cells, suggesting reduced DNA degradation in these cells. Overexpression of TatD revealed more TUNEL-positive parasites (Gannavaram and Debrabant 2012).

All organisms mentioned above possess mitochondria and an endonuclease G homolog, in contrast to *E. histolytica*. In several studies, endonuclease G is reported to interact with the TatD nuclease. When PCD was induced in *Leishmania*, the TatD-like nuclease formed complexes with endonuclease G, which were then translocated into the nucleus where the complex was responsible for DNA degradation. In addition, endonuclease G formed separate complexes with FEN-1 in *L. donovani* (BoseDasgupta et al. 2008). Similarly, Gannavaram and Debrabant (2012) suggested *T. brucei* TatD to form a DNA degradation complex with endonuclease G in the nucleus. Lacking endonuclease G, this interaction is not possible in *E. histolytica*, which raises the question of the function of the TatD protein in this parasite again.

Possible future experiments

Definitively, functional nucleases are present in *E. histolytica*, as shown in a preliminary experiment presented in the submitted manuscript (Fig. 1, TatD manuscript). Therefore, the possible activity of TatD could be re-investigated in an independent way by generating a recombinant protein with a suitable tag that does not co-purify with *E. coli* nucleases, and further purification steps could be performed. Then, it would be interesting to examine the TatD residue N200, specific for *Entamoeba* spp. Through the proximity to the active site, this residue would be likely to have an influence on any activity. However, as it remains questionable that the TatD protein is the DNase responsible for DNA damage after metronidazole treatment, other nucleases like the LINE endonuclease could play a role. The LINE endonuclease is the only DNase characterized in amoebae so far (Mandal et al. 2004), but its role regarding the DNA degradation upon metronidazole treatment is not known. Interestingly, in qRT-PCR analysis, the mRNA coding for the LINE nuclease was up-regulated after 1 and 4 h of metronidazole treatment, though not significantly after 1 h (Fig. 8,

TatD manuscript). Therefore, the LINE endonuclease should also be considered for future experiments on DNA degradation in metronidazole treated trophozoites.

9.5 Conclusion

Taking a serious look at the results of the experiments performed until now, we now have major doubts concerning the characterization of the *E. histolytica* TatD as a DNase and its responsibility for DNA damage in metronidazole treated trophozoites. So the aim to identify the DNase responsible for DNA damage in *E. histolytica* trophozoites after metronidazole treatment was not achieved. The last set of experiments revealed a major potential artefact, definitively any recombinant protein isolated by metal chelate chromatography will be contaminated by DNase activity originating from *E. coli*. This artefact could also play a role in other studies on the DNase activity of TatD or other putative DNases which used the same purification protocol. It will be necessary to identify the contaminating DNases from *E. coli* first, and then to study if *E. histolytica* TatD can be separated successfully from these contaminants.
Taken together, the question of the function of the TatD protein in *E. histolytica* still remains unanswered, but represents an interesting area of research for the future.

10. Overall discussion

In this thesis, two different chemical stress conditions in *E. histolytica* were studied. In the first part, nutrient stress in amoebae is addressed. Here, we describe the results if glucose is abruptly replaced by fructose as an alternative energy source in the culture medium. The *E. histolytica* fructokinase essential for this switch is characterized on the biochemical level.

One serious stress condition for amoebae is triggered through the nitroimidazole drug metronidazole, with death of the trophozoites as the final consequence. In metronidazole-treated *E. histolytica*, DNA damage is observed *in vitro*, but it is not known which agent is responsible for this damage. One hypothesis suggests *E. histolytica* nucleases as the cause for DNA degradation. Therefore, one aim of this study was the identification of such DNases. We describe the search for DNase genes and the discovery and molecular characterization of a homolog of the bacterial TatD DNase. As described above, the recombinant TatD could be produced but its apparent DNase activity and therefore its role in metronidazole-induced DNA damage could not be verified and further studies are needed.

Fructose as an alternative energy source for *E. histolytica* trophozoites

In the published manuscript “Molecular and biochemical characterization of *Entamoeba histolytica* fructokinase”, we report that fructose can be utilized by the amoebae as an alternative energy source to glucose. *In vitro*, the amoebae can cope with nutrient stress regarding a lack of glucose easily if fructose is present. This seems especially important for the survival of the parasite in the large intestine where only small amounts of glucose are available. For the entry of fructose into glycolysis, which represents the most important energy source for the microaerophilic amoebae, a fructokinase is essential. Therefore, dealing with a lack of glucose, the fructokinase enzyme plays an important role for the trophozoites. With the characterization of the *E. histolytica* fructokinase, new insight into the metabolism of this parasite has been gained.

E. histolytica trophozoites are usually cultured in TYI-S-33 medium (Diamond et al. 1978), which contains glucose as the only defined carbohydrate component. For our experiments, a stable *E. histolytica* culture grown in fructose containing medium, instead of glucose, was established. By now, the amoebae have been kept in fructose culture medium for more than 12 months without any negative consequences. Interestingly, the amoebae were able to adapt to the fructose medium immediately. Therefore, an alternative fructose containing culture medium for the growth of *E. histolytica* trophozoites can be suggested.

Previously, two *E. histolytica* hexokinases were studied in this laboratory, but none of them was able to phosphorylate fructose (Kroschewski et al. 2000). Now, a database search revealed a gene in the *E. histolytica* genome encoding a fructokinase homolog similar to bacterial fructokinases. This gene product was expressed in *E. coli*. Kinetic parameters of the enzyme were measured and the fructokinase mRNA expression was analyzed when amoebae were switched from glucose to fructose culture medium. Moreover, the enzyme was localized by confocal immunofluorescence, which showed a cytoplasmic distribution of the protein (Fig. 4 in manuscript).

In a coupled assay for fructose phosphorylation activity, the ADP generated in the fructokinase reaction was analyzed spectrophotometrically via measurements of the decrease of NADH. Highest activity was observed with 1 mM fructose at a temperature optimum of 37°C (Fig. 3A). Interestingly, the addition of 5 mM fructose resulted in a decreased activity to only half of that observed with 1 mM. Renz and Stitt (1993) also found a substrate inhibition with higher fructose concentrations in the fructokinase from potatoes. The formation of fructose-6-phosphate was verified spectrophotometrically in a coupled assay with glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase via the formation of NADPH. In glycolysis, fructose-6-phosphate is directly used for the generation of energy.

The activity was Mg^{2+}-dependent with an optimum concentration of 10 mM $MgCl_2$. No activity was observed with the addition of $CaCl_2$, whereas a decreased fructokinase activity was detected with $MnCl_2$ replacing $MgCl_2$. Similar observations were reported from other bacterial fructokinases with Mg^{2+} as the primarily required bivalent ion (Table 2).

The K_m of *E. histolytica* fructokinase for fructose was 0.156 mM, and the calculated V_{max} was 131.25 units/mg protein (Fig. 3B). Whereas the V_{max} was comparable to what was found in other bacterial fructokinases, the K_m value was lowest in *E. histolytica* (Table 2). Thus, the amoebic fructokinase is also active at lower fructose concentrations.

The putative phosphorylation of glucose, mannose and galactose by the recombinant fructokinase was also examined (Table 1). Towards mannose, limited substrate specificity was found, no activity was observed using glucose or galactose. Similarly, some bacterial fructokinases also showed mannose-phosphorylating activity (Scopes et al. 1985; Thompson et al. 1991; Sato et al. 1993; Table 2). Moreover, fructokinase activity was examined in lysates of fructose adapted *E. histolytica* via measurements of NADPH formation. Calculated activity in the lysate using 1 mM fructose was 26.3 units/mg protein with a calculated turnover number of 14.3 molecules per second. The fructokinase activity was about 3-fold higher in fructose adapted amoebae compared to trophozoites cultured with glucose containing medium. Interestingly, similar observations were made in bacterial studies on fructokinase activity. In *Zymomonas mobilis*, an increase of around 2-fold was observed when bacteria were grown on fructose (Zembrzuski et al. 1992). Overall, quite a few similarities were found in the bacterial fructokinases (Table 2), not surprisingly, as the fructokinase gene was probably acquired from bacteria through lateral gene transfer (Loftus et al. 2005).

Compared to amoebae grown in glucose, qRT-PCR demonstrated a significant up-regulation of the fructokinase mRNA in *E. histolytica* adapted to fructose (Fig. 2). The same picture was seen in trophozoites cultured with fructose for 2 h and 4 h, respectively. However, the highest mRNA up-regulation, which was observed after 4 h of cultivation in fructose-medium, was only around 2-fold. Therefore, the change in expression was less than the activity increase of fructokinase. This has an important consequence for other chemical stress situations: even low mRNA expression changes are relevant. For example, only modest expression changes were found after the treatment of *E. histolytica* with metronidazole (Tazreiter et al. 2008), but these changes should be regarded as physiologically significant.

Stress response of *E. histolytica* trophozoites upon metronidazole treatment

For the examination of stress responses caused by metronidazole treatment of *E. histolytica* trophozoites, preliminary experiments with untreated *E. histolytica* lysates were performed. With pUC19 plasmid used as substrate, a general endonuclease activity was found, showing nicked and linear forms of the plasmid DNA (Fig. 1, TatD manuscript). Longer incubation times and the addition of $MgCl_2$ increased the activity. Importantly, the activity originated in the amoebae and was different from the DNase activity present in the bovine serum supplement of the culture medium (data not shown). These results supported the hypothesis for the presence of DNases in amoebae which could potentially be responsible for DNA damage upon metronidazole treatment.

Some preliminary attempts to enrich and identify the DNase activity were made. *E. histolytica* extract was fractionated by anion exchange chromatography and the eluate fractions were further analyzed by means of the beacon fluorescence assay, used later to investigate purified TatD. To further separate fractions of high DNase activity, two-dimensional gel electrophoresis was performed as described previously (Leitsch et al. 2005). Spots were excised and the proteins were analyzed by tandem mass spectrometry. Unfortunately, no DNase was among the identified proteins (data not shown) and a switch to an alternative approach became necessary.

The availability of the *E. histolytica* genome (Loftus et al. 2005; Lorenzi et al. 2010) allowed the search for *E. histolytica* nucleases in the NCBI database. In total, 38 genes coding for putative nucleases were identified (Supplementary Table S1, TatD manuscript), many of which predicted to be involved in DNA repair, but no homologs for DNases I or II, endonuclease G, or caspase-dependent DNases were found. Focusing on predicted endonucleases, 16 putative hits were selected for analysis. The only endonuclease in *E. histolytica* described to play a role in apoptotic DNA degradation was the TatD protein. Described as apoptotic nuclease in several species, like in *C. elegans* (Parrish and Xue 2003), *S. cerevisiae* (Qiu et al. 2005), *Leishmania* (BoseDasgupta et al. 2008) and *T. brucei* (Gannavaram and Debrabant 2012), it was tentatively expected to find such a function of the TatD homolog in *E. histolytica* as well.

In qRT-PCR experiments, mRNA expression levels of the selected 16 putative endonucleases were analyzed. Basal expression levels were compared with the levels after 50 µM metronidazole treatment of the *E. histolytica* cells for 1 h and 4 h, respectively.

After one hour, there was a tendency towards down-regulation of the nuclease genes which, after four hours, turned into a majority of up-regulated genes (Fig. 8, TatD manuscript). Overall, 8 genes revealed significant changes, including the TatD protein. Two genes belonging to the endonuclease / exonuclease / phosphatase family and a DNA repair endonuclease were up-regulated about two-fold after 4 h of metronidazole treatment.

Whereas at the beginning TatD appeared to be a main candidate for DNA destruction in metronidazole-treated *E. histolytica*, several results, discussed in detail above, raised serious doubts about this hypothesis. First, the mRNA coding for TatD decreased rather than increased in metronidazole-treated amoebae. Second, the purified recombinant TatD contained DNase activity from *E. coli*, so the function of TatD as a nuclease has to be questioned definitively. Third, the recombinant TatD was unable to generate TUNEL-positive cells on slides. As observed by confocal immunofluorescence, TatD was found in some nuclei of metronidazole-treated amoebae, but this was not very convincing as the cells were quite damaged already.

The endonuclease encoded in a LINE (long interspersed element) was also up-regulated about 2.5-fold. Of all the genes with significant mRNA changes, the LINE endonuclease represents the most interesting case. Being the first DNase described in *E. histolytica* (Mandal et al. 2004), this enzyme now represents the best candidate of an endonuclease possibly involved in DNA damage after metronidazole treatment. However, the research on the LINE endonuclease will present a number of difficulties as well. The NCBI database does not even contain one annotated LINE endonuclease, but in the genome of *E. histolytica* HM-1:IMSS as presented in amoebaDB (Harb and Roos 2015), there is a huge number of 709 similar open reading frames (expect value ≤1e-10). So far, nothing is known, neither how many of these are translated nor what is their function in the amoebae.

11. Overall conclusions

Dealing with stress responses of the human enteric parasite *E. histolytica*, this work addressed two different aspects of metabolism. In the fructokinase study, it was shown that glucose can be replaced by fructose in the culture medium, importantly not causing any harm for the trophozoites. With the characterization of the *E. histolytica* fructokinase, a bacterial-type sugar kinase, an important gap in our understanding of the metabolism of this parasite was filled. Whereas fructose is not accepted by the *E. histolytica* hexokinases, it is readily phosphorylated and introduced into the glycolytic pathway by the specific fructokinase. If glucose is absent, fructose serves perfectly as an alternative to gain energy, which could play a role in individuals with fructose malabsorption. This study also showed that the fructokinase gene, which was most likely acquired by lateral gene transfer from bacteria, functions excellently, so *in vitro*, the switch from glucose as the primary source of energy to fructose as an alternative does not cause any serious nutrition stress in *E. histolytica*.

The second part of this work addressed the activity of metronidazole, the gold standard drug against invasive amoebiasis. Focusing on DNA damage in amoebae triggered by this drug, the identification of a nuclease responsible for this degradation was one aim of this project. *E. histolytica* possesses only one candidate endonuclease for an association with apoptotic chromosomal DNA fragmentation, this TatD protein was analyzed. However, this project turned out to be very complex. The standard metal chelate purification of the recombinant TatD co-purifies *E. coli* DNases, so the doubts remain that *E. histolytica* TatD has any endonuclease activity of its own. Generation of various point mutants of TatD did not provide a conclusive answer. So more work needs to be invested into TatD, either by using a different affinity tag or by performing further purification steps to generate preparations free of *E. coli* nuclease contaminants.
Whereas TatD mRNA was down-regulated in metronidazole-treated *E. histolytica*, the mRNA coding for the so-called LINE endonuclease was up-

regulated. The LINEs (long interspersed elements) are repeated hundreds of times in the *E. histolytica* genome, so there is also a large family of genes coding for this nuclease activity. The LINE endonuclease could be another candidate for an involvement in DNA fragmentation in amoebae upon metronidazole treatment, but this will be another complex project.

Overall, new insights into chemical stress responses of this major human pathogen were gained. Both the TatD protein and the fructokinase have been investigated in *E. histolytica* for the first time.
Still, not enough is known about the activity of metronidazole in amoebae, however, this could become very important if metronidazole-resistant *E. histolytica* strains emerge or if metronidazole may have to be taken from the market due to its significant carcinogenic and mutagenic potential. With the examination of metabolic capabilities of amoebae, it may be possible to pinpoint specific new drug targets in the future.

12. Abstract

The human enteric parasite *Entamoeba histolytica* is the cause of invasive amoebiasis and affects millions of people worldwide. In this thesis, two different metabolic stress conditions in *E. histolytica* were investigated. One stress condition the amoebae have to deal with is the variation of nutrients. In the first study, it was shown that the trophozoites can easily adapt from medium containing glucose, which is the standard carbohydrate component in the culture medium, to medium containing fructose instead. Therefore, fructose can serve as an alternative energy source for the parasite if glucose is not available. To enter fructose into the glycolytic pathway, a fructokinase is essential, as the parasite's hexokinases are not able to perform this function. Thus, the *E. histolytica* fructokinase, a bacterial-type sugar kinase, was cloned and expressed in *Escherichia coli*. The recombinant fructokinase phosphorylated fructose and mannose, but not glucose or galactose, and showed a magnesium-dependent fructose 6-kinase activity. Confocal immunofluorescence microscopy revealed a cytoplasmic distribution in the amoebae, and the fructokinase mRNA was up-regulated about two-fold in amoebae shifted to a fructose culture medium. Although it was only a modest mRNA up-regulation, this study provided an example, that an *E. histolytica* gene, which was probably acquired by lateral transfer from bacteria, can have a major regulated metabolic function in the amoebae.

In the second part of this thesis, the focus was directed to stress triggered by the treatment of *E. histolytica* trophozoites with metronidazole. This is the gold standard drug to treat amoebiasis, but its mode of action is not understood completely. One aspect is the DNA degradation in the parasite, which was shown by TUNEL assays of metronidazole-treated amoebae. It was hypothesized that the DNA damage is not caused chemically by metronidazole metabolites, but enzymatically by the parasite's DNases. After showing that *E. histolytica* extracts had DNase activity, the databases were searched, but no homologs of endonuclease G, caspase-dependent DNase, or DNases I or II were found. However, a homolog of a TatD nuclease, which was discovered in bacteria first, was identified. TatD was implicated in programmed cell death

of trypanosomes and was characterized as an apoptotic nuclease in several other organisms. Therefore, the *E. histolytica* gene was cloned, and TatD was expressed in *E. coli* with a histidine tag and purified by standard metal chelate chromatography. A magnesium-dependent DNase activity was observed at first. Quantitative RT-PCR showed a down-regulation of the TatD mRNA after metronidazole treatment and site-directed mutagenesis of conserved residues in TatD failed to reveal large changes in activity. Moreover, a second purification step by cation exchange chromatography was performed, but no enrichment of DNase activity was observed. Then we observed DNase activity in recombinant fructokinase prepared by metal chelate affinity chromatography as well. Therefore, a contamination of these preparations with *E. coli* DNases was most likely.

Taken together, it is highly doubtful that the *E. histolytica* TatD really is a DNase, which stands in contrast to what has been found in other related organisms. Structural *in silico* analysis of the protein revealed no easy solution for this problem and more work needs to be done to answer the question of the function of the amoebic TatD. Further experiments to generate preparations free of *E. coli* nuclease contaminants are needed, which could provide answers to this question in the future.

In contrast to the TatD protein, two DNA repair nucleases and an endonuclease encoded in a long interspersed element (LINE) were up-regulated up to 2.5-fold on the mRNA level after four hours metronidazole treatment. These endonucleases could be other candidates for an involvement in DNA fragmentation in amoebae upon metronidazole treatment.

With the amoebic fructokinase and the TatD protein, two interesting proteins were characterized in this thesis. Unfortunately, the identification of an *E. histolytica* nuclease implicated in DNA damage after metronidazole treatment was not successful so far. The TatD protein appeared to be an interesting candidate at first, however, most likely, it does not possess any DNase activity.

13. Zusammenfassung

Der im menschlichen Verdauungstrakt lebende Erreger *Entamoeba histolytica* ist die Ursache für invasive Amöbiasis, von der weltweit Millionen von Menschen betroffen sind. In dieser Arbeit wurden zwei verschiedene metabolische Stressbedingungen in Amöben analysiert, wobei eine dieser Stressbedingungen die Nährstoffversorgung der Amöben betrifft. In dieser ersten Studie wurde gezeigt, dass die Trophozoiten sich ohne Probleme an ein Kulturmedium anpassen können, das statt des Standardzuckers Glukose, Fruktose als Alternative enthält. Daher kann Fruktose als alternative Energiequelle dienen, wenn Glukose nicht zur Verfügung steht. Da die parasiteneigenen Hexokinasen Fruktose nicht phosphorylieren können, ist eine funktionelle Fruktokinase notwendig, um die Fruktose der Glykolyse zuzuführen. Daher wurde die *E. histolytica* Fruktokinase, die Fruktokinasen aus Bakterien ähnelt, kloniert und in *Escherichia coli* exprimiert. Das rekombinante Enzym phosphorylierte Fruktose und Mannose, nicht aber Glukose oder Galaktose. Es zeigte sich eine magnesiumabhängige Fruktose 6-Kinase Aktivität und die konfokale Immunfluoreszenzmikroskopie ergab eine zytoplasmatische Verteilung in den Amöben. Die Expression der Fruktokinase mRNA verdoppelte sich ungefähr in den Amöben, die in ein Fruktosemedium transferiert worden waren. Obwohl diese Hochregulierung der mRNA eher gering ausfiel, ist diese Studie ein Beispiel dafür, dass ein *E. histolytica* Gen, das sehr wahrscheinlich durch lateralen Gentransfer von Bakterien gewonnen wurde, eine wichtige regulierte metabolische Funktion haben kann.

Im zweiten Teil dieser Arbeit wurde untersucht, wie sich Stress, der durch die Behandlung der Trophozoiten mit Metronidazol ausgelöst wird, auf die Amöben auswirkt. Metronidazol wird als Standardmedikament für die Behandlung von Amöbenruhr verwendet, allerdings ist dessen Funktionsweise noch nicht restlos geklärt. Ein Aspekt betrifft den DNA Abbau in diesem Parasiten, der mit TUNEL Experimenten von Metronidazol-behandelten Amöben nachgewiesen wurde. Eine Hypothese ist, dass dieser Abbau der DNA enzymatisch durch amöbeneigene DNasen ausgelöst wird und nicht chemisch durch Meta-

boliten von Metronidazol. Nachdem gezeigt werden konnte, dass in *E. histolytica* Extrakten DNase Aktivität vorhanden ist, wurde eine Datenbanksuche durchgeführt. Es wurden allerdings keine Homologen für Endonuklease G, Caspase-abhängige DNase oder DNase I und II gefunden. Es konnte jedoch ein Homologes einer ursprünglich in Bakterien gefundenen TatD Nuklease identifiziert werden. TatD soll eine Rolle beim programmierten Zelltod in Trypanosomen spielen und wurde bei verschiedenen Organismen als apoptotische Nuklease charakterisiert. Daher wurde das *E. histolytica* Gen kloniert, TatD dann mit einem Histidin-Tag in *E. coli* exprimiert und mit klassischer Metallchelatchromatographie gereinigt. Zu Beginn wurde eine Magnesium-abhängige DNase Aktivität beobachtet. Die quantitative RT-PCR zeigte jedoch eine Herunterregulierung der TatD mRNA nach der Behandlung der Amöben mit Metronidazol. Das Mutieren von konservierten Resten in TatD ergab keine großen Aktivitätsänderungen, die eigentlich zu erwarten gewesen wären. Darüber hinaus wurde ein zweiter Reinigungsschritt in Form von Kationenaustauschchromatographie durchgeführt, wobei keine Anreicherung der DNase Aktivität beobachtet werden konnte. Weiters wurde auch eine DNase Aktivität bei der rekombinanten Fruktokinase festgestellt, die ebenfalls mit Metallchelatchromatographie gereinigt worden war. Daher ist anzunehmen, dass eine Kontamination der Präparationen mit *E. coli* DNasen die Ursache für die zuerst beobachtete Aktivität war. Es ist also eher unwahrscheinlich, dass das *E. histolytica* TatD wirklich eine DNase ist, was im Gegensatz dazu steht, was TatD Studien bei anderen verwandten Arten gezeigt haben. Auch *in silico* Strukturanalysen des Proteins ergaben keine einfachen Lösungen für dieses Problem. Um die Funktion von TatD in *E. histolytica* zu klären, sind daher weitere Experimente notwendig. So könnten Präparationen, die frei von *E. coli* DNasen sind, dabei helfen, Antworten zu finden.

Im Gegensatz zu dem TatD Protein zeigten zwei DNA Reparaturnukleasen und die LINE (long interspersed element) Endonuklease eine bis zu 2,5-fache Hochregulierung der mRNA nach einer 4-stündigen Behandlung mit Metronidazol. Diese Endonukleasen könnten beim Abbau der Amöben-DNA nach Metronidazolbehandlung auch eine Rolle spielen.

In dieser Arbeit wurden mit der *E. histolytica* Fruktokinase und dem TatD Protein zwei interessante Proteine charakterisiert. Leider konnte bisher keine Nuklease, die beim Abbau der DNA in Amöben nach der Behandlung mit Metronidazol involviert ist, identifiziert werden. TatD erschien am Anfang als ein sehr guter Kandidat für diese Aufgabe, allerdings sprechen die bisherigen Ergebnisse eher dagegen, dass TatD eine DNase ist.

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15. Curriculum Vitae

Personal Data

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Date of Birth: 30.10.1981
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Education

Since March 2010	**PhD student in the field of life sciences (Biology)** at the University of Vienna
Nov. 2008 - Feb. 2009	**Internship** in Bangladesh (laboratory work)
Aug. - Oct. 2008	**Laboratory work** at the Department of Specific Prophylaxis and Tropical Medicine (Medical University of Vienna)
July 2008	**Graduation to Master of Science** (with honors)
2006 - 2008	**Diploma thesis** at the Department of Behavioural Biology: "Influence of habitat characteristics and human factors on population structure, behaviour and stress of European ground squirrels (*Spermophilus citellus*)"
2001 - 2008	**Studies of Biology/Zoology** (Behavioural Biology) at the University of Vienna
2000	**High school graduation** (with honors)
1991 - 2000	BRG-Schoren, Dornbirn

Work Experience

April 2014 - 2015	Minor employment at the Department of Specific Prophylaxis and Tropical Medicine (Medical University of Vienna)

March 2010 - 2014	PhD student at the University of Vienna, employed by the Medical University of Vienna at the Department of Specific Prophylaxis and Tropical Medicine (Project funded by the Austrian Science Fund: *Entamoeba histolytica* thioredoxin, thioredoxin reductase and small thiols: interactions and role in the molecular mechanism of metronidazole toxicity)
Sept. 2009 - Feb. 2010	Self-employed in the business of data processing
April - July 2009	Part time job: Lavée Cosmetic
2006 - 2008	Part time job in data processing: Wirtschaftsauskunftei Wisur
July/Aug. 2004 - 2006	Internship: Inatura Dornbirn
1996 - 2003	Diverse vacation jobs

Publications

- Matt J, Duchêne M (2015) Molecular and biochemical characterization of *Entamoeba histolytica* fructokinase. Parasitol Res. 114(5): 1939-1947.
- Matt J, Schlosser S, Guillén N, Duchêne M (2015) *Entamoeba histolytica*: Molecular Characterisation of a DNase Homologous to Bacterial TatD. [submitted to PLoS Negl Trop Dis. on 2014 Aug 7]
- Fuehrer HP, Swoboda P, Harl J, Starzengruber P, Habler VE, Bloeschl I, Haque R, Matt J, Khan WA, Noedl H (2014) High prevalence and genetic diversity of *Plasmodium malariae* and no evidence of *P. knowlesi* in Bangladesh. Parasitol Res. 113(4): 1537-1543.
- Swoboda P, Fuehrer HP, Ley B, Starzengruber P, Ley-Thriemer K, Jung M, Matt J, Fally MA, Mueller MK, Reismann JA, Haque R, Khan WA, Noedl H (2014) Evidence of a major reservoir of non-malarial febrile diseases in malaria-endemic regions of Bangladesh. Am J Trop Med Hyg. 90(2): 377-382.
- Fuehrer HP, Starzengruber P, Swoboda P, Khan WA, Matt J, Ley B, Thriemer K, Haque R, Yunus EB, Hossain SM, Walochnik J, Noedl H (2010) Indigenous *Plasmodium ovale malaria* in Bangladesh. Am. J. Trop. Med. Hyg. 83(1): 75-78.

Scientific Presentations

- Matt J, Schlosser S, Duchêne M (2013) TatD as a possible cause for DNA degradation in *Entamoeba histolytica* after metronidazole treatment. 47. Jahrestagung der Österr. Gesellschaft für Tropenmedizin, Parasitologie und Migrationsmedizin, Wien.

- Matt J, Schlosser S, Baumann V, Duchêne M (2013) Metronidazole and DNA degradation in *Entamoeba histolytica*. XVII Seminar on Amebiasis, Merida, Mexiko.

- Matt J, Duchêne M (2012) Identification of *Entamoeba histolytica* DNases. EMBO Global Lecture Course and Symposium on Amoebiasis, New Delhi, Khajuraho, India.

- Matt J, Duchêne M (2011) DNA damage in *Entamoeba histolytica* after metronidazole treatment - could it be caused by parasite DNases? VI European Congress of Protistology (ECOP), Berlin, Germany.

- Matt J, Duchêne M (2010) Characterization of DNase activity in *Entamoeba histolytica*. 44. Jahrestagung der Österr. Gesellschaft für Tropenmedizin und Parasitologie, Graz.

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16. Acknowledgements

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